Comprehensive proteomic study of *Bacillus amyloliquefaciens* strain FZB42 and its response to plant root exudates

Dissertation

zur Erlangung des akademischen Grades

doctor rerum naturalium

(Dr. rer. nat.)

im Fach Biologie

eingereicht an der

Mathematisch-Naturwissenschaftlichen Fakultät I
der Humboldt-Univesität zu Berlin

von

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Tag der mündlichen Prüfung: 21.08.12

Summary

Bacillus amyloliquefaciens strain FZB42 is a free-living bacterium that competitively colonizes plant roots and stimulates plant growth by many different modes of action. The molecular basis of singular beneficial effects that this Plant Growth-Promoting Rhizobacteria (PGPR) exert on their hosts have been studied. To decipher the molecular cross-talk of *B. amyloliquefaciens* and its' host plants as a whole system, an extensive proteomic approach was performed.

Reference maps of the extracellular and cytosolic protein fractions were established. The highest number of secreted proteins was observed during stationary growth phase. Identified extracellular proteins belong to different functional classes, with the most prominent classes involved in carbohydrate degradation and transportation of molecules across the cell wall. Cytosolic extracts obtained from cultures grown in 1C and minimal media subjected to the 2 Dimensional Electrophoresis (2 DE), revealed 461 and 245 different protein entries, respectively. Created reference maps were subsequently used to identify proteins and processes involved in the interaction with plants, prior to exposure of bacteria to maize (Zea mays L.) root exudates.

The proteomics of two strains lacking expression of genes coding for global transcriptional regulators (degU, abrB) and four sigma factors (sigB, sigM, sigV, and sigX) were also investigated, in order to analyse their involvement in bacterial responses to root exudates.

In summary, this is the first study presenting comprehensive proteomics of Gram-positive PGPR, evaluating at the same time changes in protein expression caused by addition of root exudates at the extracellular and cytosolic level.

Key words: Proteomics, Bacillus amyloliquefaciens FZB42, 2 dimensional gel electrophoresis, root exudates, Plant Growth-Promoting Rhizobacteria (PGPR).

Zusammenfassung

Bacillus amyloliquefaciens FZB42 ist ein frei lebendes Bakterium, das Pflanzenwurzeln besiedelt und das Pflanzenwachstum durch viele verschiedene Wirkmechanismen anregt. In dieser Arbeit wurden die molekularen Grundlagen dieser positiven Wirkungen, die dieses „Pflanzenwachstum fördernde Rhizobakterium“ (PGPR) auf seine Wirte ausübt, untersucht. Um den gegenseitigen Austausch von *B. amyloliquefaciens* und seinen Wirtspflanzen zu entschlüsseln, wurden umfangreiche Proteomstudien durchgeführt. Es wurden Referenzkarten der extrazellulären und zytosolischen Proteinfraktionen erstellt.

Die größte Anzahl an ausgeschiedenen Proteinen konnte während der stationären Phase beobachtet werden. Die identifizierten extrazellulären Proteine gehören verschiedenen Funktionsklassen an, wobei die prominentesten Klassen am Kohlenhydrat-Abbau und den Transport von Molekülen durch die Zellwand beteiligt sind. Die zytosolischen Extrakte von Kulturen, die in 1C-Medium bzw. Mineralmedium angezogen wurden, und in der zweidimensionalen Gelelektrophorese (2 DE) aufgetrennt wurden, ergaben 461 und 245 verschiedene Protein-Einträge.

Die erstellten Referenz-Karten wurden anschließend verwendet, um Proteine und Prozesse, in an der Interaktion mit Pflanzen beteiligt sind, zu identifizieren. Dafür wurden die Bakterien Wurzelexudaten von Mais (*Zea mays* L.) ausgesetzt.

Die Proteine aus zwei Stämmen, denen die globalen Transkriptionsregulatoren (Degu, AbrB) und vier Sigma-Faktoren (SigB, SigM, SigV, und SigX) fehlen, wurden ebenfalls untersucht, um ihre Beteiligung an den bakteriellen Reaktionen auf die Wurzelausscheidungen zu analysieren.

Zusammenfassend ist dies die erste Studie, die umfangreiche Proteomdaten von Gram-positiven PGPR präsentiert, wobei gleichzeitig die Veränderung der Expression von extrazellulären und zytoplasmatischen Proteinen, nach Zugabe von Wurzelexudaten, ausgewertet wurde.

Schlagwörter: Proteomik, Bacillus amyloliquefaciens FZB42, 2D-Gelelektrophorese, Wurzelexudaten, Pflanzenwachstum fördernde Rhizobakterium.

1 Introduction

1.1 Plant-microbe interactions

The evolution of terrestrial plants was a driving force for microbes to explore the soil for nutrients and water (Hartmann *et al.* 2009). Plant roots produce an array of compounds, changing chemically and biologically with the environment in their vicinity. The exuded root products (Bais *et al*. 2006; Haichar *et al*. 2008) and deposition of root-derived compounds in-to the soil provides an external source of carbon, and therefore influences the microbial community structurally and functionally. Plants are able to release up to 20% of their photosynthesis products into soil, which allows the establishment of interactions with microorganisms (Haichar *et al*. 2008). For heterotrophic microbes that rely on external carbon sources the rhizosphere offers a variety of nutritional compounds. Composition of root exudates reflect the evolution or specific adaptation to abiotic and environmental conditions, for instance deficiencies in macro- and micro-nutrients (Crowley and Rengel 1999; Carvalhais *et al.* 2011). Additionally, exudates contain signaling compounds that attract specific microbial populations. Therefore, microbes have developed the machinery to sense signaling molecules, and further elicit signal transduction cascades that mediate responses to the environment (Brencic and Winans 2005). Not surprisingly, in comparison with the bulk soil, biomass and activity of microbes are enhanced in the vicinity of roots, because of exudation. This phenomenon is called a “rhizosphere effect” (Hiltner 1904). At the same time, the amount of the rhizodeposits decreases with growing distance from the root surface, mainly due to the slow diffusion and microbial activity. The diffusion range largely depends on the physical properties of soil, influencing the migration of the ions and molecular compounds, determining a concentration gradient in the soil (Hinsinger 1998). Among the plant-derived substances present in the soil, soluble compounds of root exudates have the highest diffusion range, and therefore shape the “rhizosphere extension” - a distance from the root surface that is effected by input of easily available carbon and energy sources (Sauer *et al*. 2006). The diffusion of root exudates varies with the plant species, however, the distance is calculated in only a dozen of millimeters.

1.2 Plant-root colonization

The interactions between plants and microbes can have a neutral, beneficial, or detrimental effect on the plant-host. Regardless of the outcome of the plant-microbe relationship, the colonization strategy is highly similar. In general, colonization is comprised of the following steps: recognition, adherence, invasion (in case of endophytes and pathogens), colonization and establishment of interactions (Hartmann *et al.* 2009). The recognition and motility toward plant derivate compounds are mechanisms that determine colonization (de Weert *et al.* 2002; Yaryura *et al.* 2008). Some compounds of the root exudates such as amino acids, organic acids or sugars, induce flagellum-driven chemotaxis of bacteria toward roots. Establishment of the microbe-plant interactions is initiated by the attachment of bacteria to plant roots (Rodriguez-Navarro *et al.* 2007) and the formation of biofilm - an aggregate of microorganisms embedded in the self-produced mainly extracellular polysaccharide matrix (Costerton *et al.* 1995). This well-studied mechanism of attachment of rhizobia to the root surface of legumes is hypothesized to prelude the invasion and nodulation steps. Lectins, which are proteins synthesized by several plants, bind to the bacterial Ca^{2+}-binding protein (rhicadhesin) and bacterial surface polysaccharides, resulting in secretion of bacterial cellulose fibrils, causing a formation of tight microbial aggregates on the root surface (Rodriguez-Navarro *et al.* 2007).

The release of exudates was observed in the root hair zone, sub-apical zone, and at emerging sites of secondary ramifications of roots (Hale *et al*. 1978; Curl and Truelove 1986). Not surprisingly, these sites are the favorite for plant associated root colonizing rhizobacteria (Fan *et al.* 2011). Additionally, increased numbers of formed micro-colonies was observed in the junctions between epidermal cells (Chin-A-Woeng *et al*.1997) and sites where epidermal cells were damaged - providing a rich source of nutrient (Schonwitz and Ziegler 1988).

1.3 Plant-derived compounds in the soil and their function

The soil surrounding roots is rich in plant-derived compounds. This ubiquitous phenomenon of releasing compounds by living plants is called rhizodeposition, and includes a wide range of processes. In general, rhizodeposition can be divided into two different actions: i) leakage of compounds over which the plant exerts little or no control, such as lysis and loss of cap, border and death cells, resulting in a passive loss of root exudates; and ii) exudation of specific compounds with a specific function, over which the plant exerts control (Jones *et al.* 2009). It is clear that deposition of plant tissues and cells in the soil is the main constituent of soil organic matter (Haichar *et al.* 2008). Nevertheless, water solu-

ble, diffusible, and readily available organic compounds of root exudates are of most interest for rhizobacteria (Kraffczyk *et al.*1984). Previous studies using ^{14}C labeling techniques have shown that microbial metabolites exert stimulatory effect on the exudation rate of plant roots (Meharg and Killham 1995; Kuzyakov *et al.*2003). The root exudates mainly consist of simple and complex sugars, amino acids, organic acids, extracellular enzymes, phenolics, vitamins, nitrogenous macromolecules (purines and nucleosides), and inorganic and gaseous molecules such as HCO_3^-, OH^-, H^+, CO_2 and H_2 (Uren and Reisenauer 1988; Dakora and Philips 2002; Hartmann *et al.*2009).

It is generally assumed that compounds present in the root exudates serve as a carbon source for microbes present in the soil. However, it is still unclear, which of exuded components are the primary sources of carbon and energy. Since sugars are found to be major components of exudates (Farrar *et al.*2003), with maize root exudates comprised of 65% of sugars, 33% organic acids, and 2% amino acids (Baudoin *et al.* 2003), they might be the driving force for microbial chemotaxis and root colonization. Another plausible carbon source for microbial symbionts could be root mucilage, which is secreted from the root cap, consisting of 95 – 97% polysaccharides (Bacic *et al*. 1986; Knee *et al.* 2001; Ma *et al*. 2010). The sugar residues of mucilage are bound via glycosidic linkages, which are almost as complex as linkages among sugars in the various polysaccharides of plant cell walls (Zablackis *et al.* 1995). It has been proven that soil bacterium, *Rhizobium leguminosarum* 8401, is capable of utilizing purified root mucilage as a sole carbon source for growth (Knee *et al.* 2001).

Exudation is species dependent and varies in different environmental conditions (Lambers *et al.* 2009). Root exudates produced by plants grown under iron deficiency contain higher amounts of carboxylates (mainly citrate and malate), chelating phenols, and Fe^{III} reductants; in case of dicotyledonous and non-graminaceous monocotyledonous plants; or phytosiderophores like mugineic acid, which is secreted by grasses. To overcome phosphate limiting conditions, many plants enhance rates of carboxylate exudation, phenolic compounds (probably with antimicrobial activity), and enzymes: glucanases and chitinases involved in the degradation of fungal cell walls (Weisskopf *et al.* 2006; Lambers *et al.* 2009).

1.4 Mechanisms of exudation

There are three main mechanisms: diffusion, transport via ion channels and vesicle transport that are employed by plants to release root exudates (Bertin *et al.* 2003). Release of low-molecular weight substances such as sugars, amino acids, carboxylic acids, and phenolics occurs via diffusion - a passive process of transportation along the steep concentration gradient between the cytoplasm of root

cells and the soil (Neumann 2000). Active exudation of high amounts of specific carboxylates, such as malate and citrate, in response to stress conditions (nutritional deficiencies or toxicity caused by heavy metals) is mediated by ion channels (Neumann 2000). High-molecular weight compounds including complex polysaccharides such as, mucilage and exoenzymes (e.g. acid phosphatase, phytase and peroxidase), are secreted via vesicular transport (Battey and Blackbourn 1993).

1.5 Plant associated bacteria and their use in agriculture

The term "Plant Growth-Promoting Rhizobacterium" (PGPR) describes non-pathogenic bacteria, which posses the ability to colonize root surfaces and exert a beneficial effect on plants (Kloepper *et al.* 1980). Over the years, several mechanisms of rhizobacterial growth promotion have been documented including: i) the ability to fix atmospheric nitrogen and acquisition of growth limiting nutrients (phosphorous and iron) through secretion of siderophores and organic acids (Vessey 2003) ii) synthesis of hormones such as: auxins, gibberellins, cytokinins, ethylene, that influence plant development (Lugtenberg and Kamilova 2009) and iii) suppression of plant pathogens by synthesis of compounds with antimicrobial and/or antifungal activity (biocontrol), or by mobilization of the plant to better defend itself via synthesis of compounds that elicit induced systemic resistance (ISR) (van Loon 2007). Interactions of plants with PGPR have found increased germination rates, root growth, yield (including grain), leaf area, chlorophyll content, magnesium content, nitrogen content, protein content, shoot and root weights, and delayed leaf senescence (Lucy *et al*. 2004). Additionally, PGPR are employed as agents enhancing efficiency of fertilizers (Kloepper *et al.* 2004), and as rhizoremediators degrading soil pollutants (Kuiper *et al.* 2004). The above mentioned modes of actions can be combined, and a single PGPR will display several abilities to exert beneficial effects on plant. Not surprisingly, possibilities to use PGPR in agriculture and horticulture as biofertilizers, rhizoremediators, phytostimulators and stress controlling agents have attracted worldwide attention.

A few decades ago, scientists hypothesized about the use of microorganisms, which could compete with plant soil pathogens, particularly by producing antibiotic compounds. Since then, a large body of research has been made in order to elucidate beneficial actions of PGPR on plants, with the biocontrol properties of Gram-negative *Pseudomonas fluorescens,* strains CHA0 or Pf-5 the best understood. Although fluorescent *Pseudomonas* spp. have greatly contributed to the understanding of mechanisms involved in promotion of plant growth in wide aspects, their use as a commercial product has a disadvantage from an application point of view (Francis *et al.* 2010). *Pseudomonas* spp. as an asporogenous bacteria, lose their viability after storage over period of several weeks. Alternatively, species of *Bacillus* and related Gram-positive genera, have much better shelf life and form spores,

which facilitates the development of commercial PGPR-based products that posses better storage ability (Haas and Defago 2005). Additionally, several physiological traits of spore forming bacteria, such as production of a multilayered cell wall structures, and secretion of peptide antibiotics, peptide signal molecules, and an array of extracellular enzymes, allow these bacteria to inhabit diverse ecosystems (McSpadden Gardener 2004).

The physiology of Gram-positive bacteria make them particularly attractive for use in agriculture as inoculants. *Bacillus* spp. are known to improve growth conditions of many plants by different modes of action, for instance, by inducing systemic resistance against sugar beet fungal pathogen (Bargabus *et al*. 2003; Bargabus *et al.* 2004), against *Cucumber mosaic* virus (Zehnder *et al.* 2000), and oomycets (Yan *et al.* 2002) on tomato. Positive effects of *Bacillus* spp. on sunflower (Forchetti *et al*. 2010) and chickpea (Wani and Khan 2010) have also been reported.

To fulfill demands for improved crop yield, soil productivity, and reduction of the application rate of mineral fertilizers, there is a need to exploit PGPR for sustainable agriculture. As environmentally friendly microbial agents have a potential to substitute conventionally used chemical agents. Consequently, commercialized products containing PGPR of different genera including *Acinetobacter, Agrobacterium, Arthrobacter, Azospirillum, Bacillus, Bradyrhizobium, Frankia, Pseudomonas, Rhizobium, Serratia,* and *Thiobacillus* have been developed. These products containing PGPR can be applied by introduction into soil, seeds, roots, tubers or other planting materials to enhance the crop growth (Choudhary and Johri 2009). The application of biocontrol PGPR agents under green-house conditions was successful, while the field trials were not so promising. Several studies done in the past suggested ineffectiveness of a single PGPR strain inoculation (Egamberdiyeva and Hoeflich 2004; Lucy *et al.* 2004), but co-inoculation of different strains have provided more consistency (Ryu *et al*. 2007).

1.6 What makes *B. amyloliquefaciens* FZB42 a good PGPR

Among *Bacillus* species, *B. amyloliquefaciens* was originally characterized by its ability to produce huge amounts of α-amylase, an enzyme necessary for liquefaction of starch (Ingle and Erickson 1978), with soil being it's natural habitat. An ecotype of *Bacillus amyloliquefaciens* has been isolated from the rhizosphere and been shown to exert beneficial effects on various plants, such as soybean (Buensanteai, 2008a, Buensanteai 2008b) and maize (Idriss *et al*. 2002). Additionally, suppression effect on root-knot nematode infection has been reported (Burkett-Cadena *et al.* 2008).

Bacillus amyloliquefaciens strain FZB42 was recently extensively studied. Commercially available agents, based on the FZB42 as a PGPR, have been successfully applied in field trails. Once the whole

genome became available (Chen *et al*. 2007), series of investigations aimed to elucidate bacterial mechanisms responsible for plant growth promotion and biocontrol activities were undertaken. Recently proving that FZB42 is able to effectively colonize plant roots (Fan *et al.* 2011), and positively affect plant growth by: i) production of substances with auxin-like activity, which enhance plant growth (Idris *et al*. 2007); ii) secretion of phytase, an extracellular enzyme involved in utilization of phytate phosphorus which provides nourishment of the plant under conditions of phosphate limitation (Idriss *et al.* 2002); iii) production of iron chelating siderophore bacillibactin, enabling FZB42 cells to accumulate and take up limited iron ions from their natural environment (Chen *et al*. 2009). Biocontrol activity is mainly related to the suppression of plant pathogens within a rhizosphere, caused by synthesis of secondary metabolites such as: lipopeptides active against phytopathogenic fungi (bacillomycin and fengycin); antibacterial acting polyketides (bacillaene, macrolactin and difficidin); the dipeptide bacilysin, which is active against a wide range of bacteria and yeast *Candida albicans*; antimicrobial and antiviral heptapeptide surfactin (Koumoutsi *et al*. 2004; Chen *et al*. 2006; Koumoutsi *et al*. 2007; Moldenhauer *et al*. 2007; Schneider *et al*. 2007).

B. amyloliquefaciens FZB42, as a soil saprophytic bacterium, uses plant cell walls as a nutrient. Synthesis of extensive repertoires of degradative extracellular enzymes reflects the chemical complexity of the composite substrate. Indeed, many bacteria accommodate to the subtle differences of the plant cell walls polysaccharide structure found in their natural habitat, acquire a large number of closely related degradative enzymes during the evolution (Tailford *et al*. 2009).

1.7 Proteome analysis

While significant progress in elucidating the details of plant-microbe interactions has been made in recent years, many fundamental questions remain unresolved. The need for a broad spectrum tool induced development of techniques that give a possibility to study detailed response of plants and bacteria to various treatments and to one another as a whole system (Cheng *et al.* 2010). Proteomics is one of the methods that meet this requirements.

In general, proteomics is the study of protein properties including the elucidation of structure and sequence, post-translational modifications, stability, cellular localization, determination of proteins copies number per cell and potential interaction partners, on a large scale to obtain a global view of a cellular processes (Blackstock and Weir 1999). The term ‘proteome’ was coined for the first time in 1994 by M. R. Wilkins and K. Williams, at the 2-D electrophoresis conference, and describes the protein complement of the genome (Wilkins *et al.* 1996). Opposite to the static genome, the proteome behaves

dynamically depending on the cell or tissue type, external influences (e.g., chemical, physical, or physiological stress), and the analogy of both is only superficial. While genome sequencing is a feasible, to decipher the proteome is an infinite task. The proteome is thought to be an order of magnitude more complex than the genome, due to the numerous modifications (e.g., phosphorylation, glycosylation, acetylation, sulphation) or variation of translation start or stop sites or by frame shifting during which a different set of triplet codons in the mRNA is translated (Fields 2001).

The complexity of the proteome requires a method that is capable of visualising several thousands of proteins. Two-dimensional electrophoresis fulfils such requirements, and provides a powerful tool for the separation of the complex proteins forming the proteome (Cheng *et al*. 2010). During the 1970s O'Farrell developed the classical 2-D electrophoresis system (O'Farrell 1975). Carrier ampholytes were used to create a pH gradient in a tube gel system for separation in the first dimension according to the protein isoelectric point. Subsequently, SDS PolyAcrylamide Gel Electrophoresis (PAGE) was employed to separate proteins according to their molecular weight (O'Farrell 1975). However, application of the carrier ampholytes was met with limitation. Therefore Immobilized pH Gradient (IPG) was developed, which is generated by a limited number (6-8) of well-defined chemicals co-polymerized with the acrylamide matrix. This development optimized the isoelectric focusing in the first step of 2-D electrophoresis (Bjellqvist *et al.* 1982). Nowadays, commercially available pre-casted IPG strips can be applied in broad scientific communities, with previously observed problems of discrepancies in pattern reproducibility between laboratories not as evident (Corbett *et al.* 1994).

The most significant breakthrough in the proteomic approach was the improvement at the level of a protein's identification. The development of high throughput mass spectrometry methods: Electrospray Ionisation (ESI) and Matrix Assisted Laser Desorption/Ionization (MALDI) led to a rapid progression in proteomics (Karas and Hillenkamp 1988; Fenn *et al.* 1989). Additionally, the growing number of completely sequenced genomes, e.g., first sequenced bacterium *Haemophilus influenza* (Fleischmann *et al.* 1995), first eukaryotic sequence *Saccharomyces cerevisiae* (Goffeau *et al.* 1997), and human genome (Venter *et al.* 2001); generated a blueprint of all gene products of a specific organism.

1.8 Gene regulation in Bacillus

Adaptability is the crucial ability of bacteria to prosper and survive in a wide variety of environmental conditions. Thus, to respond and better adapt to altered conditions, bacteria have a large reservoir of genetic information to cope with environmental stimuli (Tetsch and Jung 2009). Recognition of exter-

nal signals and its conversion in-to transcriptional or behavioral responses is essential for the survival of bacteria (Fabret *et al.* 1999).

1.8.1 Transition state regulators

Bacteria prioritize the activation of proteins necessary for utilization of alternate carbon sources when nutrients are low, leading to, effective competition with other species for scarce resources. During the transition state, bacteria activate the production of extracellular proteases and degradative enzymes, and additionally, switch on the alternative pathways to seek for and transport the nutrient under limitation. To be even more competitive, some bacteria pump out antibiotics and antimicrobials (Phillips and Strauch 2002). In nature, bacterial growth is balanced between transition and stationary states, mainly because of the insufficient amount of nutrients. Thus, intricate networks of regulatory mechanisms direct regulation of gene expression to maintain this balance. External stimuli activate the alternative RNA polymerase sigma factors that modulate expression of proteins with adaptive and survival functions (Phillips and Strauch 2002). Various two-component signal transduction systems sense deteriorating growth conditions and activate expression of appropriate genes. A large number of global regulatory DNA binding proteins provide a link between external conditions and the physiological state of bacteria.

1.8.1.1 DegS-DegU two-component regulation system

Similarly to *B. subtilis, B. amyloliquefaciens* is subjected to drastic variations in environmental conditions. The DegS-DegU two-component regulation system is involved in adaptation to harsh conditions. The recognition and conversion of external signals in-to chemical response is performed by DegS, a membrane-associated protein exhibiting bifunctional activities: kinase and phosphatase. After autophosporylation, DegS transfers the phosphoryl group to a cognate response regulator DegU (Msadek *et al.* 1990; Tanaka *et al.* 1991).

DegU plays a pivotal role in the regulation of various post exponential phase processes, including activation and inhibition of genetic competence, motility and biofilm formation, (especially induction of exopolymer production), as well as activation of degradative enzyme production (Murray *et al.* 2009). However, DegU has regulatory activities in both unphosphorylated and phosphorylated states, which led to its designation as a ‘molecular switch’ (Dahl *et al.* 1992). The level of phosphorylated DegU form (DegU~P) is indirectly dependent on the quorum sensing responsive transcription factor

ComA, which increases with cell density and is maximal at stationary phase (Dervyn *et al.* 2004; Murray *et al.* 2009). At the onset of stationary phase (when most of the readily available nutrient is depleted), DegU~P activates the transcription of degradative enzymes, including an intracellular protease and several secreted enzymes: levansucrase, proteases, α-amylase, β-glucanase(s), and xylanase, providing bacteria sugars or amino acids to replenish intracellular pools. On the other hand, unphosphorylated DegU activates genetic competence through the recruitment of ComK (Hamoen *et al.* 2000).

1.8.1.2 *AbrB global regulator*

The AbrB protein belongs to a group of proteins called Transition State Regulators (TSRs), which control the functions activated in the state between vegetative growth and the onset to stationary phase and sporulation (Perego and Hoch 1988). AbrB effects expression of more than 100 genes (Chumsakul *et al.* 2010) involved in post-exponential functions, such as the production of antibiotics, formation of biofilms, development of competence, initiation of sporulation, production of extracellular proteases and other degradative enzymes and cannibalistic behavior (Makarewicz *et al.* 2008). In *Bacillus anthracis*, expression of toxin genes has also been noted (Saile and Koehler 2002). Expression of *abrB* is not essential and its mutation does not affect cells viability under laboratory conditions (Phillips and Strauch 2002). However, in the natural environment, AbrB could possibly play an essential role in adapting to changing conditions and survival. The genes demonstrated to be negatively controlled by AbrB belong to the group mainly involved in sporulation (*spo0E, spoVG*), development of competence (*ComK*), and synthesis of extracellular protease *aprE* (Strauch and Hoch 1993). There is evidence that AbrB positively affects expression of the transition-state-regulator Hpr, as well as genes involved in histidine utilization (*hutP*), and arginine biosynthesis (*argC*). However, the nature of the interaction is still unclear, as AbrB may bind directly to the DNA, or the activation may be due to repression of yet another unidentified repressor (Strauch and Hoch 1993).

Expression of AbrB is growth-phase dependent, and occurs during the lag and exponential phase, which is kept at a narrow threshold range of regulatory effectiveness by its negative autoregulation. At the onset of transition state, phosphorylated Spo0A directly represses *abrB* transcription, which results in intracellular level dropping below the threshold of effectiveness, resulting in the AbrB dependent regulatory effects being lifted (Phillips and Strauch 2002).

Despite the fact that AbrB plays an essential role in reorganization of gene expression, it is still unclear how AbrB selects DNA binding sites, and exerts desired functions. The search for identification of consensus AbrB-binding site selection and recognition failed (Strauch 1995; Xu and Strauch 1996).

It has been hypothesized that AbrB (tetramer form) requires a specific three-dimensional architecture of DNA helix to bind (Sullivan *et al.* 2008).

1.8.1.3 *Hpr global regulator*

Similar to AbrB, Hpr is a Transition State Regulator (TRS) and plays a major role in adaptation of the cell to suboptimal growth conditions. Hpr protein has been reported to regulate expression of proteases, cell responses to oxidative stress during exponential growth (Phillips and Strauch 2002), production of bacilysin in *B. subtilis* (Inaoka *et al*. 2009) and *B. amyloliquefaciens* (Mariappan unpublished results), and catabolite repression of sporulation (Shafikhani *et al.* 2003).

According to transcriptomics analysis Hpr affects expression of 560 genes. However, only a few were experimentally demonstrated to be governed by Hpr (Caldwell *et al*. 2001). Caldwell identified *yclF,* a gene with high similarity to novel di- and threepeptide transporter from *Lactococcus lactis*, as the only one positively influenced by Hpr. The regulation of *aprE* and *nprE* (extracellular proteases) expression by Hpr occurs via direct binding to the target DNA. Unlike AbrB, Hpr's consensus recognition site has been determined (Kallio *et al.* 1991). Hpr exerts a negative effect on gene expression of oligopeptide transport systems (*opp* and *app*).

Expression of *hpr* is positively regulated by AbrB, by the phosphorylated form of Spo0A, and possibly by autoregulation (Phillips and Strauch 2002).

1.8.2 Sigma factors

Transcription is a complex process that requires simultaneous involvement of several different proteins. RNA polymerase (RNAP) governs transcription in bacteria. The core of the RNAP enzyme consists of five subunits: beta (β), beta prime (β'), two alphas (α), omega (ω) and a additional sigma protein (σ) that binds to the enzyme and initiates transcription. Most of what is known about gene regulation by sigma factors have been derived from experiments with *E. coli*, and is generally assumed to be applicable to *B. subtilis* (although sizes of RNAP subunits differ) (Haldenwang 1995). In bacteria, gene expression is regulated either by binding of a regulatory protein directly to the DNA strand, or by the σ proteins. These σ proteins bind to the core RNA polymerase, which help the holoenzyme to recognize the promoter elements and initiate transcription at this site (Haldenwang 1995). Association of sigma factors and RNAP is transient. Generally, dissociation of sigma factor is thought to be followed by the initiation of transcription; however, in case of σ^{70} the transcriptional factor of *E. coli*,

sigma factor remains attached to the core RNAP enzyme at least during early elongation (Kapanidis *et al.* 2005). The regulon of a single sigma factor comprises of many genes, and can have well-defined primary functions, e.g., genes regulated by the sporulation sigma factors in *B. subtilis* (Piggot and Hilbert 2004), or contribute to multiple functions, such as the stationary-phase and general stress response genes regulated by sigma B in *Listeria monocytogenes* (Kazmierczak *et al.* 2003).

Interestingly, since RNA polymerase requires the sigma factor for specific binding to DNA, and together with the observation that single sigma factor confers promoter selectivity, it was suggested that sigma factor could directly bind to the DNA. This hypothesis was confirmed *in vitro*, and binding of sigma D (Chen and Helmann 1995) and A (Yeh *et al*. 2010) has been evidenced. However, *in vivo* gene regulation occurs with the assistance of core RNAP, because the sigma D binding specifity is too low, and sigma A is unable to discern the optimal promoter spacing.

Most of the bacterial genomes encodes for several sigma factors, which initiate transcription of genes with various classes of promoters and different consensus sequences. Thus, based on the sequence similarity, bacterial sigma factors belong to two distinct families: σ^{70} and σ^{54}. In contrary to the σ^{70} family, σ^{54} family contains only one group of sigma factors with no sequence similarity to the previous family. In addition, the initiation of transcription, despite formation of the RNAP holoenzyme and attachment to the consensus promoter site, does not start without interaction with an activator. The activator protein usually binds to the enhancer, located ~100 upstream of the transcriptional start site of a σ^{54} promoter (Studholme and Dixon 2003). Subsequently, DNA looping is required for contact of an activator with the RNAP complex, which is followed by the transcription start (Wosten 1998; Studholme and Dixon 2003). 54 sigma family factors regulate transcription of various genes thought not to be essential for growth (Merrick 1993).

The genome of *B. subtilis* encodes at least 18 sigma factors including a recently identified protein named YvrI, exhibiting divergent amino acid sequence from σ^{70} family sigma proteins (MacLellan *et al*. 2009). Exponentially growing cells utilize at least six sigma factors for transcription initiation of housekeeping genes: A, B, C, D, H and L (Huang and Helmann 1998). Additional four, E, F, G and K, which are necessary for endospore formation were identified. The complete genome sequence revealed another seven factors, which were grouped into a new extracytoplasmic function (ECF) subfamilly (Kunst *et al*. 1997). Similarly, *B. amyloliquefaciens* genome encodes for 16 sigma factors, six of which are involved in the transcription of primary genes: A, B, D, H, L, and I; with another four involved in endospore formation: E, F, G and K. Six sigma factors with putative extracytoplasmic function were identified in *B. amyloliquefaciens*, five of which have counterparts in *B. subtilis* (Chen *et al*. 2007).

1.8.2.1 *Primary function sigma factors*

The majority of sigma factors belong to the σ^{70} family, which is further subdivided into three different functionally and structurally related groups (Lonetto *et al*. 1992). Sigma factors from the first group are responsible for the transcription of most genes expressed in exponentially growing cells and are essential for cell survival (Wosten 1998). It is generally believed, that in any given eubacterium, only one sigma factor with primary functions is present (Gruber and Bryant 1997). The primary "housekeeping" sigma factor in *B. subtilis*, σ^{A}, is encoded by *rpoD* gene. Promoter consensus sequences recognized by σ^{A} have been identified as identical to the consensus sequence recognized by σ^{70} of *Escherichia coli* (Jarmer *et al.*. 2001). The sequence of sigma factors from the second group is quite similar to primary sigma factors, but genes transcribed with their assistance are not essential for cell growth.

1.8.2.2 *Alternative sigma factors*

The third group of sigma factors known as alternative, differ considerably in amino acid sequence from primary sigma factors (Wosten 1998). Genes regulated by alternative sigma factors belong to specific regulons, required during specific physiological and developmental stages. This group consists of different subgroups. Various characteristics are summarized below.

1.8.2.2.1 Flagellar sigma factors

To survive in unfavorable conditions *B. subtilis* needs a flexible regulation at gene level. One of the alternative sigma factor is the flagellar σ^{D} governing genes involved in flagella dependent motility. This regulator is active in the late exponential phase and is responsible for expression of genes required for production of a functional flagella, such as the gene encoding the flagellin filament protein (Hag), several chemotaxis proteins and cell-wall remodeling enzymes (known as autolysins) required for cell separation after division (Helmann and Moran 2002b; Veening and Kuipers 2010). Additionally, the minor extracellular protease gene *epr* was reported to be transcribed by SigD RNA polymerase (Dixit *et al*. 2002). Compilation of published and unpublished data indicated that 65 genes of *B. subtilis* are regulated by σ^{D} (Serizawa *et al*. 2004). Interestingly, recent studies suggest that beside regulation of motility, *B. subtilis* determination to either swim or to be chained cells depends on the intracellular level of SigD protein (Veening and Kuipers 2010).

1.8.2.2.2 Extracytoplasmic function (ECF) sigma factors

The extracytoplasmic sigma factors belong to the largest and most diverse subgroup of environmentally responsive transcriptional regulators of the σ^{70} family. Besides their action as general stress response proteins, ECF σ factors are also involved in transcription of regulons helping the cell to endure unfavorable environmental conditions (Helmann 2002a). Regulatory devices employed by the ECF σ factors for transduction of environmental stimuli are ubiquitous in bacteria and restricted to this group of organisms (Staron *et al*. 2009). Hence, mechanisms of signal transduction and gene regulation are common for most of ECF σ factors. In most cases, ECF σ factors autoregulate their own expression. Usually ECF genes are co-transcribed with their negative regulator, cognate anti-sigma factor, a transmembrane protein with extracytoplasmic sensory domain and intracellular σ factor inhibition activity. In the absence of a stimulus, the anti-σ factor tightly binds to the σ factor, thereby keeping it inactive (Staron *et al.* 2009). External signals trigger the inactivation process of the anti-sigma factor either by proteolytic degradation, or by changes in conformation. After the liberation of ECF σ factor from the anti-sigma factor "trap", the RNA polymerase core enzyme can recruite and again use ECF σ factor for redirecting gene expression.

Physiological functions of three of the seven ECF σ factors (σ^M, σ^W, and σ^X) encoded by the genome of *B. subtilis* have been widely investigated and described. In contrast, the inducers of ECF σ factors σ^V, σ^Z, σ^Y, σ^{YlaC}, and their functions, are still to be defined.

1.8.2.2.2.1 Sigma factor σ^X

One of the best characterized ECF sigma factors of *B. subtilis* is σ^X. SigX regulates a wide range of genes related to cell wall metabolism including *dltA* and *pssA* operons, which modulate the overall net charge of the cell envelope (Cao and Helmann 2004). Induction of σ^X is caused by various cell wall antibiotics, and stress factors affecting the cell envelope (Wiegert *et al*. 2001; Cao and Helmann 2004; Pietiainen *et al*. 2005). A *sigX* mutant is vulnerable to cationic antimicrobial peptides and has difficulties in enduring high temperature and oxidative stress (Mascher *et al*. 2007). Presence of *sigX* gene is required for proper biofilm formation, through involvement in transcription of *abh* - an abrB homolog, which positively regulates σ^X-regulated genes (Kobayashi 2007).

1.8.2.2.2.2 Sigma factor σ^W

σ^W controls expression of around 60 genes (Zellmeier *et al*. 2006), including a large fraction of the genes that are most strongly induced in response to alkali shock (Wiegert *et al.* 2001). Additional fac-

tors such as salt stress, phage infection, and addition of vancomycin to the growth medium were demonstrated to induce the σ^W regulon (Zellmeier *et al.* 2006). Interestingly, it was demonstrated that σ^W regulon provides resistance to a wide array of antimicrobial compounds synthesized by *Bacillus* strains (Butcher and Helmann 2006).

1.8.2.2.2.3 Sigma factor σ^M

The regulon of σ^M comprises of approximately 57 genes (30 operons), including genes associated with cell wall biosynthesis, shape determination and cell division, DNA damage responses, and detoxification enzymes (Eiamphungporn and Helmann 2008). Expression of the partially autoregulated sigM gene is induced by stresses. These include high salinity, ethanol, heat, acid, phosphate starvation, superoxide stress, and exposure to cell wall antibiotics such as bacitracin, vancomycin, and cationic antimicrobial peptides (Thackray and Moir 2003; Jervis *et al*. 2007). A significant portion of genes whose expression is affected by vancomycin treatment is shared between σ^M and σ^W (Cao *et al*. 2002). However, a detailed understanding of the composition, and ultimately the role of the σ^M regulon, is still lacking.

1.8.2.2.2.4 Sigma factors σ^V, σ^Z, σ^Y and σ^{YlaC}

The knowledge of σ^Y, σ^{YlaC}, σ^V and σ^Z, sigma factors is sparse. σ^Y, regulates its own operon, comprising an additional five genes assumed to encode a toxic peptide. Only one gene has been demonstrated as a σ^Y target: *ybgB*, whose product is a putative immunity protein for an antimicrobial peptide (Cao *et al*. 2003; Tojo *et al*. 2003). Ambiguous results concerning genes regulated by σ^V have been reported (Asai *et al*. 2003; Zellmeier *et al*. 2005). ECF sigma factor YlaC contributes to resistance to hydrogen peroxide (Ryu *et al*. 2006). Comparing the lists of genes putatively regulated by σ^V, there is an extensive overlap with genes that have been already shown to be under control of σ^M, σ^X, or σ^W (Zellmeier *et al.* 2005). Consequently, difficulties in determining physiological functions of some ECF sigma factors might be caused by redundancy of their functions. It is worth noting, that a quadruple mutant Δ*sigVZY* and Δ*ylaC*, subjected to the broad range of assays, does not display new phenotypes (Mascher *et al.* 2007).

1.8.2.2.3 Heat shock sigma factors

When bacteria are exposed to stress conditions, a myriad of unique General Stress Proteins (GSP) are rapidly synthesized which function as protein damage prevention and repair mechanisms. It has been

demonstrated that σ^B plays a major role in induction of general stress proteins, being the first alternative sigma factor identified in bacteria (Haldenwang and Losick 1979). Expression of the whole regulon of GSP that covers around 150 proteins is a considerable burden for the cell (Hecker *et al*. 2007). Thus, activity of SigB is controlled tightly by the anti-sigma factor (RsbW) and the anti-anti-sigma factor (RsbV). During vegetative growth, *sigB* is constitutively coexpressed with seven of its principal regulators, an operon recognized by house-keeping sigma factor σ^A. Exposure to physical (e.g. heat shock, osmotic shock, ethanol) or nutritional (e.g. azide treatment, glucose or phosphate limitation) stress conditions activate SigB, which binds to its internal promoter and causes expression of its own gene (Kuo *et al*. 2004).

1.8.2.3 *Sigma-factors involved in sporulation*

When cells experience adverse conditions, some Gram-positive bacteria, including Bacillus and Clostridium genera form endospores. These endospores are dormant, durable and temporarily non-reproductive structures, able to survive without nutrition and resistant to various physical and chemical stresses. In *B. subtilis* formation of the spores is controlled by five sigma factors σ^H, σ^E, σ^K, σ^F and σ^G.

1.9 Aims of the study

B. amyloliquefaciens strain FZB42 is a Gram-positive plant root colonizing bacterium. The multiple beneficial effects that this bacterium exerts on plants have been documented, and nowadays, with farming in many European countries and China currently utilizing its benefits through application of Rhizo®Vital 42 (ABiTEP GmbH, Berlin, Germany) - commercially available products containing spores of FZB42. Taking under consideration the physiological values of sporogenous Gram-positive bacteria, the knowledge concerning molecular basis of their interactions with plants is still limited, despite several investigations studying basis of plant-bacteria cross talk being performed. Those approaches examined only a single biochemical pathway, missing the plethora of information obtained by "OMICs" approaches. Therefore, it has been concluded that broad-spectrum approaches need to be applied in order to explore the detailed responses of bacteria to plant signaling compounds, with proteomics appearing to be a perfect method to study the system as a whole.

Most of the proteomic studies of beneficial bacteria concerned symbiotic nitrogen-fixing or phytopathogenic strains, while the issue of plant-associated, Gram-positive bacteria have not been adequately addressed. Therefore, a comprehensive proteomic study of free living PGPR FZB42 was performed.

The main objectives of this thesis were:

i) creation of a reference map of extracellular proteins secreted by FZB42, which are important in the adaptation to changing environmental conditions and establishment of interactions with other organisms, as well as the extensive characterization of those proteins.

ii) creation of *B. amyloliquefaciens* reference cytosolic proteins map which will facilitate future studies on bacterial responses to various stimuli, and better understanding of the main metabolic routes suggesting alternative applications in agriculture and environment.

iii) identifying the cytosolic and extracellular proteins that play a role in bacterial responses after exposure to maize root exudates, with reference maps necessary in this evaluation.

iv) analyzing response of extracellular and cytosolic proteins to maize root exudates in strains lacking expression of chosen global regulators and sigma factors; again, for this evaluation reference maps are necessary.

2 Results

2.1 Growth conditions of *Bacillus amyloliquefaciens* FZB42 strain for the analysis of the extracellular proteome

B. amyloliquefaciens FZB42 wild type and mutant strains deficient in the following genes: *degU, abrB, sigB, sigX, sigM*, *sigV* were cultivated aerobically at 24°C in medium 1C together with soil extract, in either presence or absence of maize (*Zea mays* L.) root exudates. Wild type cells were sampled at two time points: during the transition and early stationary growth phases (OD_{600} of 3 and 4.5, respectively) (Figure 1), while the cells of the mutants were harvested only at transition stage. Cultures were centrifuged and extracellular proteins were precipitated with trichloroacetic acid (TCA) of final concentration 10% (w/v) and subjected to 2 Dimensional Electrophoresis (2-DE).

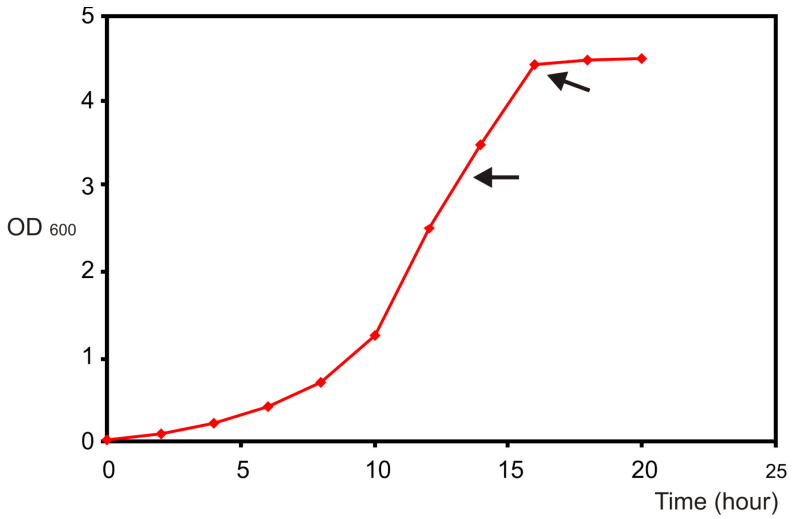


Figure 1 Growth curve of *B. amyloliquefaciens* FZB42 strain in 1C medium at 24°C. Secretory proteins were harvested at two time points indicated by arrows.

2.2 The secretome of *B. amyloliquefaciens* FZB42 predicted according to presence of signal peptides

Availability of *B. amyloliquefaciens* genome allows prediction of proteins containing the signal for translocation across the cell wall. Among the 3693 Open Reading Frames (ORFs), 289 proteins (7.2%) were predicted to be potentially exported from the cytoplasm. Theoretical secretome was created to set appropriate conditions for analysis (Figure 2). Since most of the extracellular proteins with molecular masses from 7 to 150 kDa could be clearly detected across a pH gradient ranging from 4 to 11, the IPG strips with pH gradient 3 – 10 together with a 15% polyacrylamide gel were chosen as the standard analytical window.

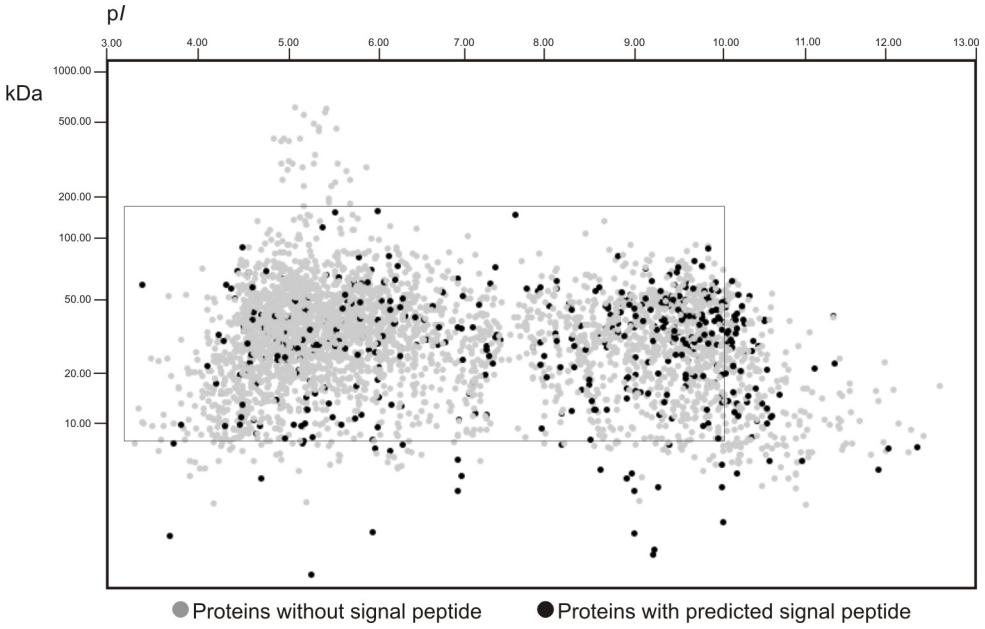


Figure 2 Theoretical secretome of *B .amyloliquefaciens* is indicated by black points. The figure represents a 2-DE gel separation of the total proteome according to calculated isoelectric point (p*I*) and molecular weight (kDa). The rectangle indicates the analytical window used in the experiments.

According to the sequence of their signal peptide most of the predicted extracellular proteins are secreted by Sec machinery pathway. Only 10 proteins were predicted to contain potential twin-arginin motifs, suggesting translocation via Tat pathway. The number of predicted twin-arginin sequences is similar to other Gram-positive bacteria such as *B. subtilis* (Tjalsma *et al*. 2004) or *Mycobacterium leprae* (Dilks *et al*. 2003). Similar to *B. subtilis* (Tjalsma *et al.* 2004), four proteins with pseudopilin

signal peptides have been identified (ComGC, ComGD, ComGE and ComGF) in *B. amyloliquefaciens*.

Among the proteins with predicted signal peptide, the majority (213) posses recognition sites for cleavage by type I signal peptidase, while 72 proteins contain signal peptides with sites for cleavage by lipoprotein-specific signal peptidase II (Figure 3).

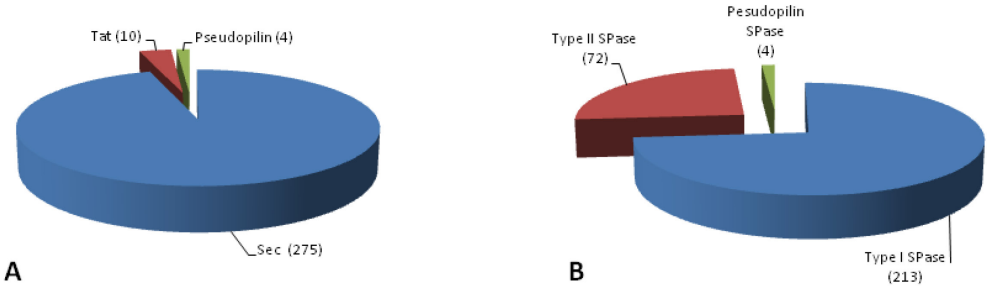
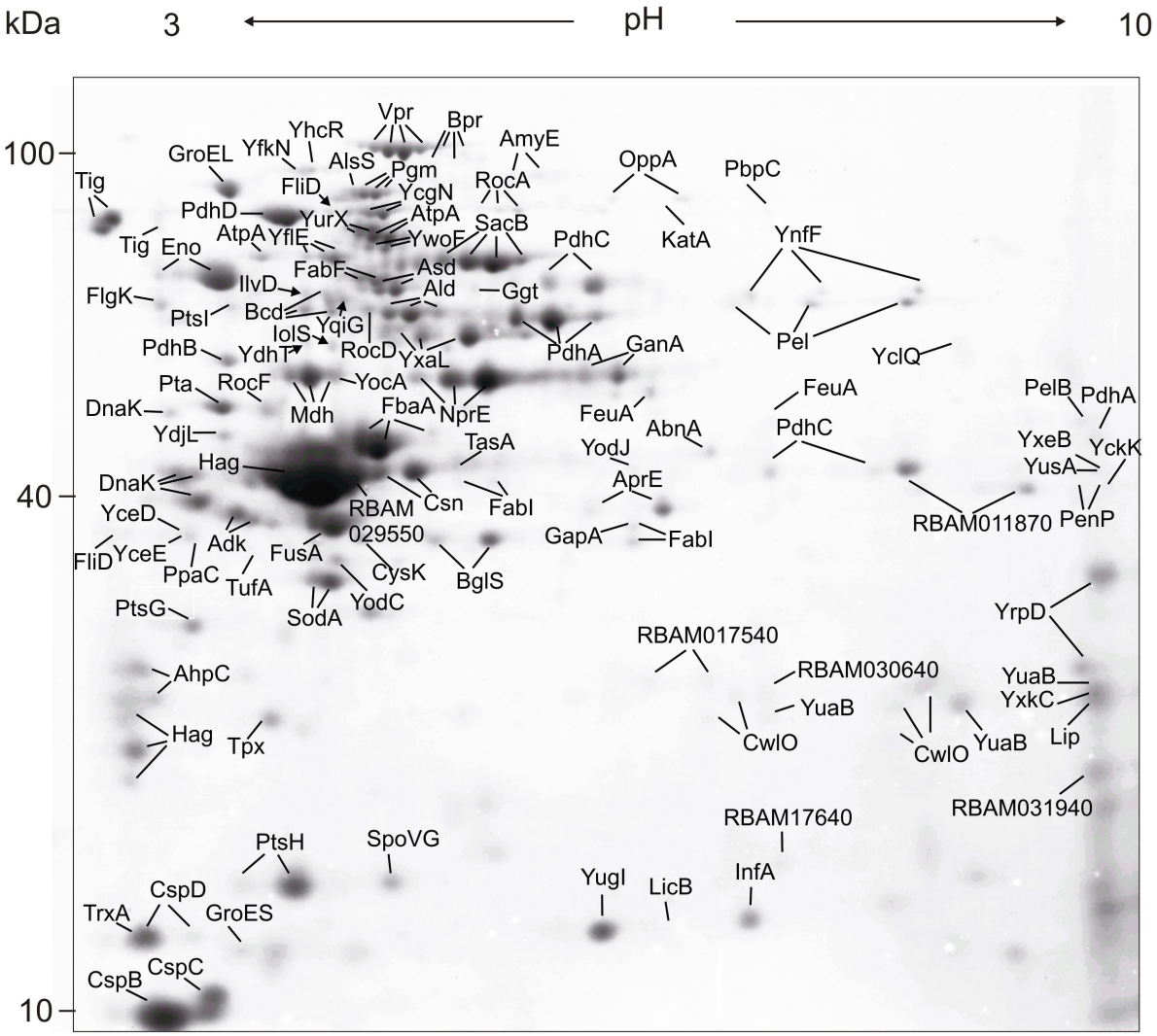


Figure 3 Prediction of the proteins with signal peptides. A: Contribution of the different secretion systems to the pie chart. The numbers in parentheses represents the number of proteins predicted to posses signal peptide directing the translocation via corresponding pathway. B: Contribution of signal peptidases to the pie chart. The numbers in parentheses are the numbers of proteins with recognition sites for the appropriate signal peptidase. Sec = Sec dependent protein secretion. Tat = twin arginine protein secretion. Pseudopilin = pseudopilin export pathway.

2.3 Reference map of the secretome of *B. amyloliquefaciens*

Due to the absence of an outer membrane, Gram-positive bacteria are able to secrete huge amounts of extracellular proteins into the growth medium. PDQest computer-assisted analysis of two dimensional gels revealed that the number of visible protein spots detected in the stationary phase (Figure 4B) was greater than in the transition phase (Figure 4A), (239 ± 22.9, 151 ± 3.8, respectively). All visible protein spots were cut out from the colloidal Coomassie stained gels. 125 proteins were identified by MALDI-TOF MS (Appendix Table 1 and 2). Some of the proteins e.g. Vpr, AmyE, SacB, occurred as multiple spots, which possibly is an artifact of isoelectric focusing due to TCA precipitation during sample preparation (Voigt *et al*. 2006).

A


kDa
3
pH
10
100
40
10
Vpr
Bpr
YhcR
AmyE
GroEL
YfkN
AlsS
Pgm
OppA
PbpC
Tig
FliD
YcgN
RocA
PdhD
YurX
AtpA
SacB
AtpA
YflE
YwoF
PdhC
KatA
YnfF
Tig
Eno
FabF
Asd
IlvD
Ald
Ggt
FlgK
PtsI
Bcd
YqiG
IolS
Pel
PdhB
YdhT
RocD
YxaL
PdhA
GanA
YclQ
Pta
RocF
YocA
FeuA
PelB
PdhA
DnaK
Mdh
FbaA
NprE
FeuA
AbnA
PdhC
YdjL
TasA
YodJ
YxeB
YckK
Hag
YusA
DnaK
AprE
YceD
RBAM
029550
Csn
FabI
RBAM011870
PenP
FliD
YceE
Adk
FusA
GapA
FabI
PpaC
TufA
CysK
BglS
PtsG
SodA
YodC
YrpD
RBAM017540
AhpC
RBAM030640
YuaB
YxkC
YuaB
Hag
Tpx
CwlO
CwlO
YuaB
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InfA
CspD
TrxA
GroES
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CspC

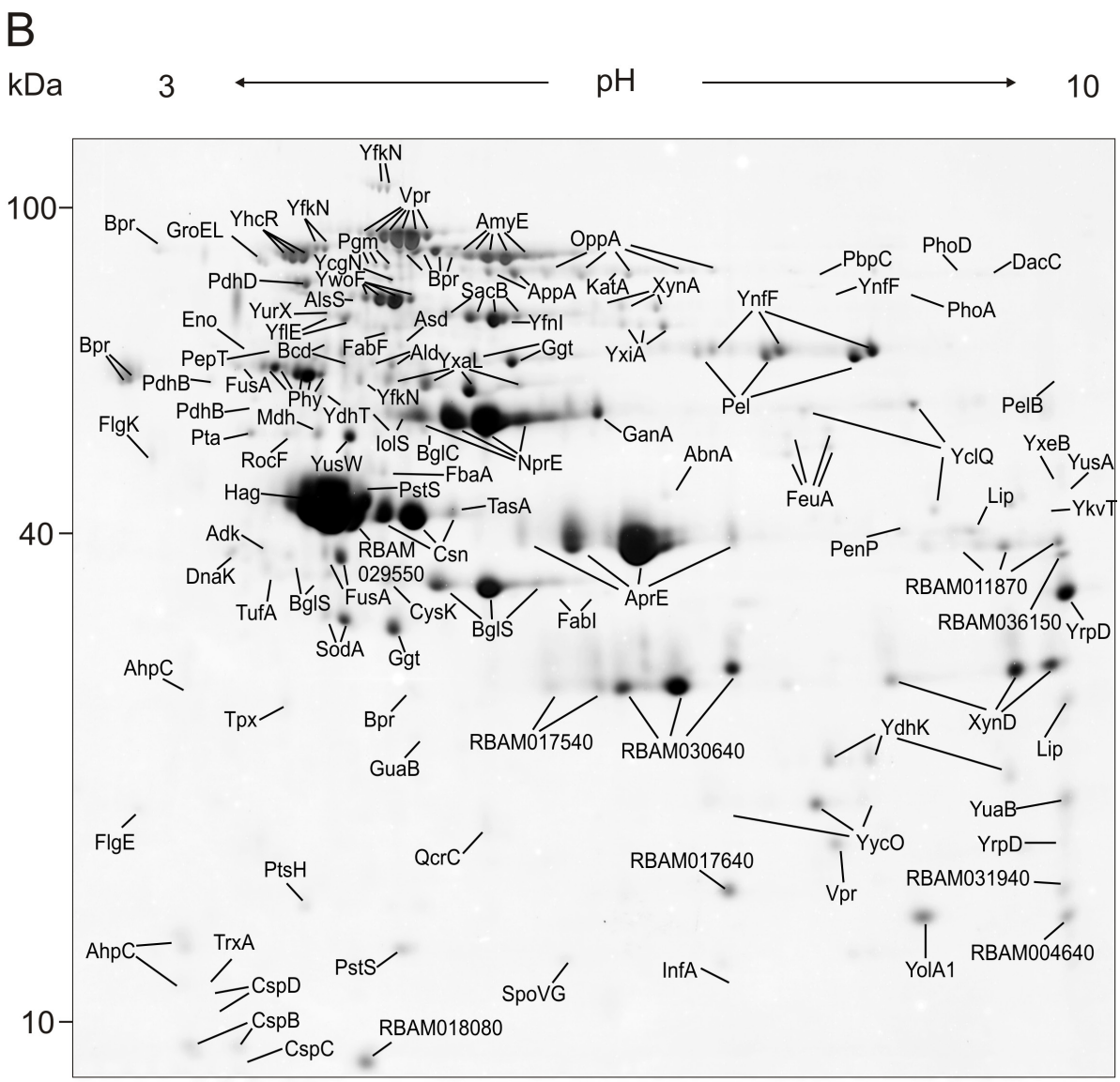

Figure 4 The extracellular proteome of *B. amyloliquefaciens* FZB42; (A) proteins secreted in to the growth medium sampled during transition phase. (B) Proteins secreted in to the growth medium sampled after entry into stationary phase.

The amino termini of the identified proteins were used to search for presence of signal peptides. Among the 125 extracellular proteins, 65, were predicted to possess a signal peptide. The N–terminal domains of many signal peptides displayed peptide type I signal peptidase cleavage sites with the consensus motif A-S-A at positions – 3 to – 1, relative to the cleavage site. This is corresponding to findings made in *B. subtilis* (Tjalsma *et al*. 2000). The N–terminal domain of 11 proteins contain so-called lipobox with the consensus sequence L-(A/S)-(A/G)-C, where the conserved cysteine residue is a target for lipid modification and the first residue of the mature lipoprotein after cleavage by signal peptidase type II (Tjalsma *et al*. 2000). Those proteins are predicted to be secreted via Sec pathway. Three proteins: PhoD (phosphodiesterase/alkaline phosphatase), YfkN (bifunctional 2',3'-cyclic nucleotide

2'-phosphodiesterase/3'-nucleotidase precursor protein), and QcrA (menaquinol-cytochrome C reductase iron-sulfur subunit) had the twin arginine motif (RR/KR) signal peptide directing the secretion through the Tat pathway. Length of the predicted signal peptides varied between 20 and 62 amino acids (Table 1). Additionally, three proteins contained transmembrane segments in the mature part, and thus were predicted to be anchored in the membrane: PtsG (glucose-specific enzyme IICBA component), RBAM011870 (putative ABC-type multidrug transport system, permease) and YocA (conserved hypothetical protein).

Table 1 Putative signal peptides of detected extracellular proteins of *B. amyloliquefaciens.*

Proteins	ID	Signal Peptide[1]	SPase I/II
CwlO	RBAM_032020	MKKSLLTLGLASVIGTSSFLIPFTKT**ASA**	
DacC	RBAM_018470	MKTYVKRLSAVILLLIIAAVPYIDDA**AKA**	
PonA	RBAM_020470	MLSLVILFVLGIVGGAAAFAVLV**SGA**	
YocH	RBAM_018960	MKKTIMSFVAVAAISTTAFGAH**ASA**	
QcrA	RBAM_020720	MGGKQDIS**RR**Q**FL**NYALTGVGGFMAASALMPMV**RFA**	
TasA	RBAM_022940	MGMKKKLSLGVASAALGLALVGGG**TWA**	
LicB	RBAM_035790	MNILLVCAAGMSTSLLVTKME**KSA**	
YxaL	RBAM_036900	MKKKTASLRMKTLAAGAAVAAALSMGAVTDLPGAKWLHPAA**AQA**	
RBAM_031940	RBAM_031940	MKKRLSWISVCLLVLFSAAGMLF**STA**	
AprE	RBAM_010500	MRGKKVWISLLFALALIFTMAFGST**SPA**	
Bpr	RBAM_015130	MKKKTRNRWK*G*SVLSAIVVSSLLFPGA**AGA**	
NprE	RBAM_014550	MGLGKKLSVAVAASFMSLTISLPG**VQA**	
Vpr	RBAM_035320	MKKGIIRYLLPAFVLSFTLSTS**SQA**	
AbnA	RBAM_025870	MKNRFRRMIRFSCAAVTAGLLLMSSSP**ASA**	
AmyE	RBAM_003280	MFEKRFKTSLLPLFAGFLLLFHLVLSGPAA**ANA**	
BglC	RBAM_018100	MKRSISIFITCLLIAVLTMGGLLPSP**GSA**	
BglS	RBAM_036190	MFYRMKRVLLLLVTGLFLSLCTITST**ASA**	
Csn	RBAM_029740	MKISLKKKAGFWKKTAVSSLIFTMFFTLMMSGT**VFA**	
GanA	RBAM_012120	MFKNRLKRVFVNAICLSLIFTAFTFFEKSPK**AKA**	
Pel	RBAM_007720	MKKMLFMLAVCLCMIPAD**VYA**	
PelB	RBAM_036320	MIKKTRHLAFLAAFGFALCLAIVCSVS**KQA**	
RBAM_018080	RBAM_018080	MRKQQAINHKGAILMLKSKIKKIAGAAVIAGALLVSVSP**AKA**	
SacB	RBAM_037650	MNIKKFAKRATVLTFTTALLAGGA**TQA**	
XynD[b]	RBAM_018150	MCKKCWVCLWVLAL*LLSC*FTG**KSA**	
YdhT	RBAM_035930	MLKKLAVCLSIVLLLLGAASP**ISA**	
YnfF	RBAM_018140	MMSSVKKTICVLLVCFTMLSVMLLGPGVTE**VSA**	
YxiA	RBAM_036390	MKTSFFRVCLVSAAVIGFTLPQ**AKA**	
Ggt	RBAM_018540	MKKKKFMNLCFIVLLSTLLAAGSIPYH**AQA**	
FabF	RBAM_011340	MSKKRVVVTGLGALSPLG**NDA**	
Lip	RBAM_003010	MKHIKNKILVVLTVCMLSVISVFAFQPTV**SKA**	
Bsn	RBAM_029600	MTKKLWFLPIVCLFFIFGWAAPSAS**AGA**	
RBAM_029550	RBAM_029550	MEGFIMRSKTKNILAVLLAGIMTFGLIIGLSQS**AQA**	
YfkN	RBAM_008030	MRIQK**RR**CS**V**NMTLRILLPLVMILSLILPTSP**IKA**	
YhcR	RBAM_009450	MKKKRGMRLTCCLLTVCFMMFSVKD**AEA**	

PhoA	RBAM_009670	MSLFKQVRSKLLPAAAVSVLTAGLIAGAGLQHS**EQA**
Phy	RBAM_019640	MNHSKTLLLTAAAGLMLTCGAVS**SQA**
Blm	RBAM_011860	MRKLSLLIAGLFLFGLFTAGMKP**AHA**
PenP	RBAM_012080	MKNKARIQFGICIGLLCLSFTGFNPLFGS**AHA**
RBAM_017540	RBAM_017540	MKGLVKAAVLTVTLGIGGAFYSSD**ASA**
RBAM_030640	RBAM_030640	MKISSLVFPVLLAAGLIPGL**PSA**
RBAM_004640	RBAM_004640	MVSERGKCSKRIISVPPKYIVFHFSLHYISKGGFPIRHAAKGILFAASLA LVTTTFVPA**SLA**
RBAM_017400	RBAM_017400	MKKKIIAGALAFGLIPLMGTAD**VSA**
RBAM_017640	RBAM_017640	MKKYWICIAVFLLCFSGIHS**AKA**
RBAM_036150	RBAM_036150	MKKAFKMMAAVLLTVLTAAPFGGFAPS**AEA**
YolA1	RBAM_002540	MKKRLVMLFAAAAAVLLLVSGLWVPS**ASA**
YrpD	RBAM_010640	MMMKKGLFAGVLTTAVLFGTCTAEIPGLISPNT**AEA**
YuaB	RBAM_028180	MKMKQKFFSTVMASLFGLVLLLSLPTA**SFA**
YwoF	RBAM_032390	MKIKLSILSAAVLAAGITAFVWPK**TEA**
YycO	RBAM_037190	MCKKLFKAVLPFALSFTMVLSFSGMN**VKA**
XynA	RBAM_033790	MFKFKKKFLVGLTAAFMSISMFSAT**ASA**
PhoD	RBAM_002930	MTHDGRLDEWIKELENKSFQNNTFD**RR**A**FI**QGAGKIAGLSLGLAIAQS IGAFE**VNA**
PbpC	RBAM_004370	MKKGLYLLLLLAGIIG**LIG**C
YodJ	RBAM_019410	MKKSTKWFSLAAALSVTAIV**GTG**C
AppA	RBAM_011380	MKLRKSALMMLSVFMAFAIF**LSA**C
FeuA	RBAM_002120	MKKLAFAFMILLLAFT**AAA**C
OppA	RBAM_011430	MKKRWSIVGLMLIFSLV**LSA**C
PstS	RBAM_023290	MKKNKWMLMLLMAAVMIV**AAA**C
YckK	RBAM_003780	MKKALFALFMMVSIAM**LAA**C
YclQ	RBAM_004080	MKKFALLFIALVTIFV**VSA**C
YxeB	RBAM_036560	MKKQSWLIGLALLLVFA**LSA**C
YusA	RBAM_029810	MKKIVLSALLLVFAGV**LAA**C
YdhK	RBAM_006150	MKNQKGKGVLEMKARLFLFSMFLLIPVLA**LAG**C

a) SignalP, LipoP and TatP were used to predict putative signal peptides. The hydrophobic H-domain is shaded grey. Residues at position -3 to -1 relative to the predicted SPase I and II cleavage site are bold and underlined. For lipoproteins the conserved cysteine residue, which is part of the lipobox and resides at the +1 positions of the mature lipid-modified lipoprotein, is also shown. Proteins with a potential twin-arginine motif in the N-domains of their signal peptides are marked RR, and the twin-arginine motif is shown in bold.

b) XynD was previously predicted as lipoprotein (Tjalsma, Bolhuis, Jongbloed, Bron and van Dijl 2000), however it is not clear, because it possesses the I SPase cleavage site. The so-called lipobox is printed in italics.

Proteins identified as being extracellular have been classified into 6 functional groups according to the SubtiList Functional Classification Code. The enzymes involved in intermediary metabolism represented the largest group with 45% of all identified proteins. These consisted of proteins involved mainly in degradation of carbohydrates derived from the plant cell wall, as well as an enzyme hydro-

lyzing chitin, the second ubiquitous polymer of fungal origin. A prominent group of "metabolic enzymes" constitute proteins involved in metabolism of amino acids, nucleotides and nucleic acids (6%).

Around 23% of the extracellular proteins were involved in dynamics of the cell wall. Several proteins in this group are known as lipoproteins, accounting for 9% of the identified proteins. These lipoproteins are expected to be anchored at the bacterial cell wall and are mainly involved in transport of oligopeptides and various metals throughout the cell wall. Other important groups of proteins were involved mainly in detoxification of oxygen radicals or survival under stress conditions.

11% of the proteins were identified as similar to hypothetical proteins occurring in *B. subtilis*, whose functions have not been assigned yet. However, being significantly identified indicates their possible function during the bacterial life cycle. Interestingly, four hypothetical proteins without similarity to already known proteins have been identified in the secretome of *B. amyloliquefaciens.*

The classical N-terminal signal peptide sequence was not found in 60 proteins among 125 identified by MALDI TOF MS. The extracellular location might indicate cell lysis during culturing or sample preparation. Some of them appeared to be abundant cytoplasmic proteins, such as the chaperone proteins GroEL and DnaK, the translation elongation factor Tu (Tuf), trigger factor (Tig), enolase (Eno) and the superoxide dismutase (SodA). Another explanation for secretion of proteins lacking signal peptide could be the existence of a Sec independent secretion pathway. Thus, SecretomeP was employed to search the sequence of proteins with nonclassical signal peptide, with 23 of them predicted to be secreted via Sec independent pathway (Appendix Table 2). Function of some of proteins such as KatA, SodA, cold-shock protein (CspD), FlgGK is consistent with secretion, as they are involved in response to environmental stress or elicitation of plant basal defense (Abramovitch *et al*. 2006).

2.4 Comparison of *B. amyloliquefaciens* extracellular proteomes at transition and stationary phases

Secretion of proteins into the growth medium differed during the cell growth stages. There were 78 proteins secreted constitutively, irregardless of the growth phase. These proteins are important components of the *B. amyloliquefaciens* secretome and play an essential role since they are present in both growth phases. Example of such proteins was flagellin (Hag), which formed a prominent spot during all tested growth phases. However, samples of extracellular proteins taken from transition phase exhibited additional flagellin spots, though its position on 2-D gel was not in agreement with calculated molecular weight and isoelectric point.

To present the changes in production of extracellular proteins throughout the growth cycle, a merged image composed of average 2-D gels representing exponential and stationary phases was created using the false-color imaging of the Delta 2D software (Decodon, Greifswald, Germany) (Figure 5). Images were overlaid and protein spots representing stationary growth are displayed in red, while green spots represent transition growth. Proteins secreted in both growth phases are indicated in yellow.

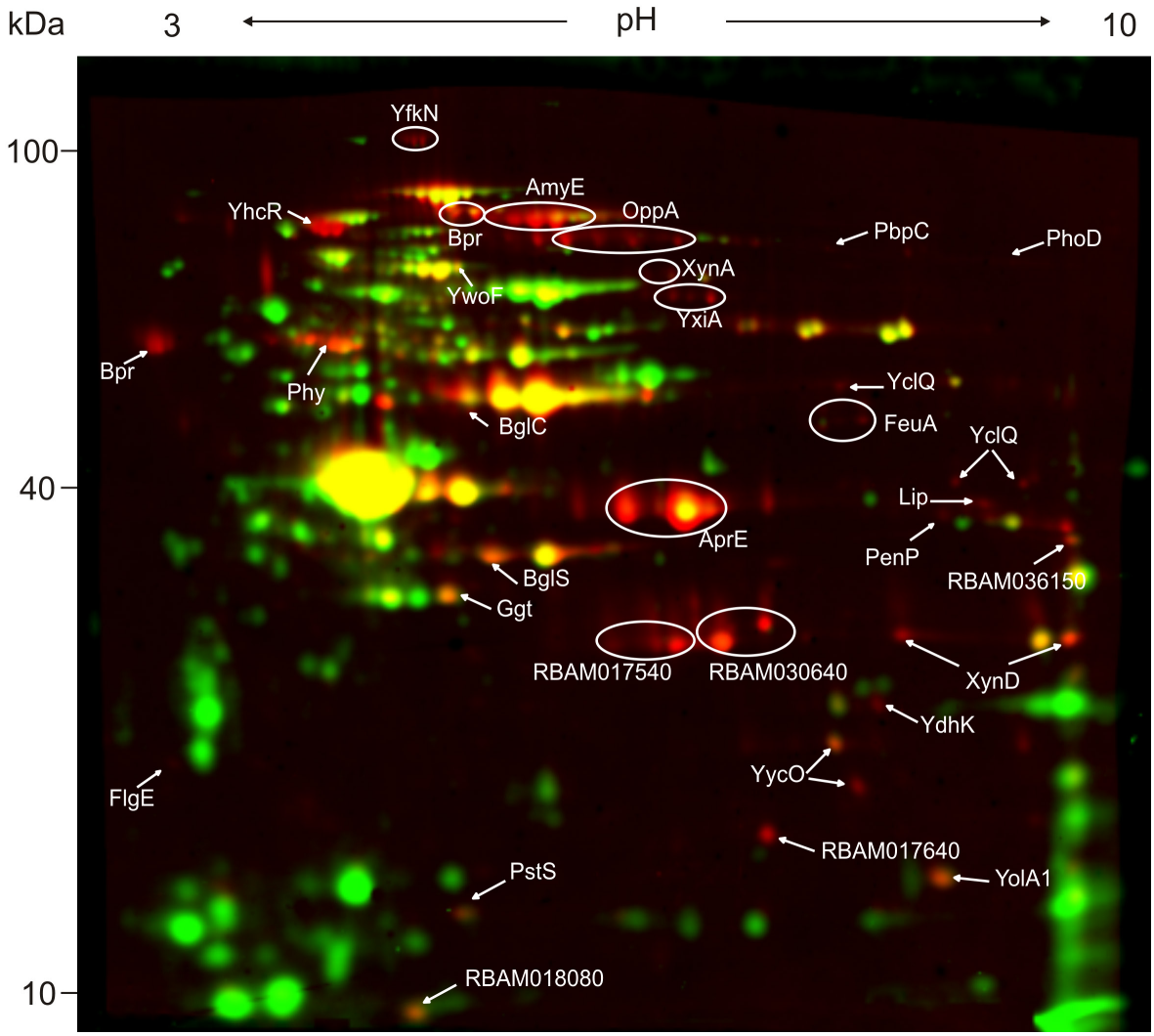

Figure 5 Dual channel image of the protein patterns of *B. amyloliquefaciens* FZB42 cells grown in 1C medium. The 2-D protein pattern of cells from the exponential phase (green) was compared to the pattern from stationary phase (red).

Comparing numerical data of spot intensities taken from two growth phases revealed 32 proteins (Table 2) present at significantly higher concentration during the stationary phase. Some of them (six proteins) belong to the group of enzymes taking part in depolymerization of highly organized sugar chains. Proteases were secreted at both growth phases, although not at the same level; Bpr and AprE were strongly induced at stationary growth phase. Interestingly, proteins involved in phosphate degra-

dation (Phy) and phosphate acquisition upon phosphate starvation (PhoD), were secreted into the growth medium only during stationary phase.

Most of the proteins that are present at low concentration or only during the transition phase belong to the group of cytoplasmic proteins (Figure 5, spots in green color). This may indicate the reduction of cell lysis during stationary phase. Additionally, the quantity of certain proteins involved in cell wall turnover and peptidoglycan hydrolysis (CwlO, YodJ), were reduced, what may suggest lowering of cell growth speed (Tjalsma *et al.* 2004).

Table 2 Extracellular proteins present at higher levels during stationary phase.

Protein with signal peptide	Function	Fold change	*p*-value
Cell wall			
PbpC	Penicillin-binding protein 3	10.00	0.025
PonA	Penicillin-binding proteins IA/IB	2.27	0.039
Membrane bioenergetics (electron transport chain and ATP synthase)			
YycO	Hypothetical protein RBAM_037190	10.00	<0.001
Mobility and chemotaxis			
FlgE	Flagellar hook protein	10.00	<0.001
Transport/binding proteins and lipoproteins			
FeuA	Iron-binding protein	6.54	0.001
OppA	Oligopeptide ABC transporter (binding protein)	6.33	0.001
PstS	Phosphate ABC transporter (binding protein)	10.00	0.001
YclQ	Putative ferrichrome ABC transporter	10.00	<0.001
Metabolism of amino acids and related molecules			
AprE	Serine alkaline protease (subtilisin)	6.57	0.001
Bpr	Bacillopeptidase F	10.00	0.010
YfkN	Putative multifunctional phosphoesterase	10.00	<0.001
Metabolism of carbohydrates and related molecules			
AmyE	Alpha-amylase	2.00	0.049
BglC	Endo-1,4-beta-glucanase	10.00	<0.001
BglS	Endo-beta-1,3-1,4 glucanase	10.00	0.001
XynA	Endo-1,4-beta-xylanase	10.00	<0.001
XynD	Endo-1,4-beta-xylanase	5.61	0.016
YolA	Hypothetical protein RBAM_002540	10.00	0.001
Metabolism of nucleotides and nucleic acids			
YhcR	Putative 5'-nucleotidase	10.00	<0.001
Metabolism of coenzymes and prosthetic groups			
Ggt	Gamma-glutamyltranspeptidase	3.10	0.049
Metabolism of lipids			
Lip	Triacylglycerol lipase	10.00	<0.001
YwoF	Hypothetical protein RBAM_032390	1.90	0.011
Metabolism of phosphate			
Phy	3-phytase precursor	10.00	<0.001
PhoD	Phosphodiesterase/alkaline phosphatase	10.00	0.048

Detoxification			
PenP	Beta-lactamase precursor	10.00	<0.001
Transposon and IS			
YxiA	Hypothetical protein RBAM_036390	10.00	0.002
Similar to unknown proteins from other organisms			
RBAM 1754	Putative chitin binding protein	2.08	0.049
RBAM 3064	Hypothetical protein RBAM_030640	10.00	<0.001
RBAM 3615	Hypothetical protein RBAM_036150	10.00	0.013
YdhK	Conserved hypothetical protein RBAM_006150	10.00	0.002
No similarity			
RBAM 1764	Hypothetical protein RBAM_017640	10.00	<0.001
RBAM 1808	Hypothetical protein RBAM_018080	1000	<0.001

Two major groups of the proteins identified during transition and stationary phases were involved in carbohydrate and amino acid metabolism. The third prominent group of proteins secreted during transition phase were proteins related to transport of oligopeptides. The number of proteins involved in detoxification was significantly higher at transition than at stationary growth phase (Table 3).

Table 3 Comparison of specific proteins for each growth phase.

Functional category	**Transition phase**	**Transition phase with SP [a]**	**Stationary phase**	**Stationary phase with SP [a]**
Cell wall	4	4	3	3
Germination	-	-	1	-
Membrane bioenergetics	2	-	4	1
Mobility and chemotaxis	3	-	3	-
Sensors (signal transduction)	1	1	-	-
Sporulation	2	1	2	1
Transport/binding proteins and lipoproteins	10	9	8	7
Protein folding	4	-	2	-
Protein modification	1	1	1	1
Protein synthesis, Elongation	2	-	2	-
Protein synthesis; Initiation	1	-	1	-
RNA modification	1	-	2	1
Metabolism of amino acids and related molecules	13	4	12	4

Metabolism of carbohydrates and related molecules	23	10	26	15
Metabolism of coenzymes and prosthetic groups	1	1	1	1
Metabolism of lipids	4	2	4	2
Metabolism of nucleotides and nucleic acids	5	4	4	3
Metabolism of phosphate	-	-	3	3
Adaptation to atypical conditions	3	-	3	-
Detoxification	8	2	5	1
Transposon and IS	1	1	-	-
Similar to unknown proteins from other organisms	-	-	2	2
No similarity	3	3	2	2
Similar to unknown proteins from *B. subtilis*	7	4	12	8
Total number of proteins	99	47	103	55

a) SP signal peptide.

2.5 Analysis of the *B. amyloliquefaciens* secretome in response to maize root exudates

Proteomics was used to identify the bacterial proteins that are differentially secreted in response to plant root exudates.

2.5.1 Differentially secreted proteins at transition phase

To better understand the beneficial effect of FZB42 on plants, comparative proteomics of extracellular proteins obtained from cultures grown in the presence or absence of maize root exudates was performed. Additionally, soil extract which is a blend of compounds derived from the breakdown processes of plant wall material and dead bacteria was added to the growth medium to mimic conditions present in the soil. It is hypothesized that growth in presence of this array of substances would require expression of proteins with novel functions.

Analysis of proteins secreted by FZB42 during transition growth phase, found 17 as differentially expressed in response to root exudates (wild type + root exudates/wild type – root exudates; WT+RE/WT-RE). More proteins (10) were found to be up rather than down-regulated (7 proteins) (Table 4). When the proteins were grouped based on the functional categories, enzymes involved in the degradation of carbohydrates had the greatest number of representatives (Figure 6).

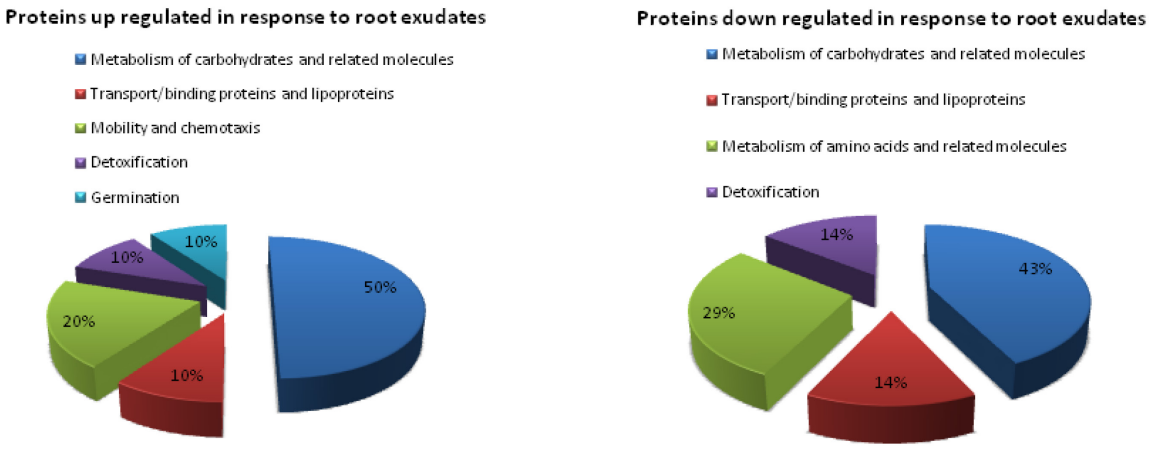

Figure 6 Contribution of functional categories to the pie chart as a percentage of differentially expressed secretory proteins isolated from cells grown at transition phase.

From the 10 proteins stimulated by presence of root exudates only four possessed a signal for secretion, two of which are missing signal peptides, however are reported as being secreted *via* an unknown pathway (Hag and FliD). The protein whose expression was strongly increased in presence of maize root exudates was acetolactate synthase (the *alsS* gene product). AlsS was secreted only when the maize exudates were added in-to the growth medium. Although AlsS is localized intracellularly, and does not possess a signal for secretion, its presence in the secretome of *B. amyloliquefaciens* FZB42 is consistent with environmental function of the protein. Ryu *et al.* (2004) reported its positive effect of induction of plant systemic resistance in other *Bacillus* spp. (Figure 7A).

Additionally, increase in secretion of proteins involved in various carbohydrate degradation processes like arabinases, glucomannanases, xylanases were also observed (AbnA, YdhT, and YnfF) (Figure 7B, C, and D, respectively). Moreover, two proteins taking part in motility and chemotaxis: flagellin (Hag) and flagellar hook-associated protein 2 (FliD) were affected by presence of root exudates (Figure 7E, F, respectively). Expression of a lipoprotein involved in transport of oligopeptides across the cell wall (OppA) was two times greater when root exudates were added to the medium (Figure 7G). In addition,

analysis of the extracellular proteome revealed three proteins without known secretion signals YqiG, Tpx, PdhC, as being significantly up-regulated in presence of root exudates (Figure 7H, I, J).

Table 4 Differentially expressed proteins in response to root exudates, isolated from transition phase.

	Protein	Function/Similarity	Fold change	*p* value[a]	Export signal[b]
	Up-regulated				
1	AlsS	Acetolactate synthase	10	0.012	-
2	YqiG	Putative NADH-dependent flavin oxidoreductase	7.15	0.010	-
3	AbnA	Arabinan-endo 1.5-alpha-L-arabinase	3.59	0.001	Sec
4	YnfF	Conserved hypothetical protein RBAM_018140	3.14	0.030	Sec
5	FliD	Flagellar hook-associated protein 2 (HAP2)	2.92	0.012	-[ex]
6	OppA	Oligopeptide ABC transporter (binding protein)	2.34	0.022	Lipo
7	Hag	Flagellin protein	2.03	0.017	-[ex]
8	PdhC	Pyruvate dehydrogenase	1.96	0.003	-
9	YdhT	β-1,4-mannanase RBAM_035930	1.62	0.036	Sec
10	Tpx	Thiol peroxidase	1.5	0.010	-
	Down-regulated				
11	Csn	Chitosanase	-1.56	0.007	Sec
12	BglS	Endo-beta-1.3-1.4 glucanase	-1.6	<0.001	Sec
13	PtsI	Phosphoenolpyruvate-protein phosphotransferase	-1.62	0.008	-
14	PdhC	Pyruvate dehydrogenase	-2.59	0.033	-
15	YcgN	1-pyrroline-5-carboxylate dehydrogenase	-3.63	0.001	-
16	RocA	3-hydroxy-1-pyrroline-5 carboxylate dehydrogenase	-6.07	0.045	-
17	YceE	Putative tellurium resistance protein	-10	<0.001	-

a) Proteins were considered significantly different when p value < 0.05.

b) Export signals are Sec-type signal peptides (Sec); lipoprotein signal peptides (Lipo); - proteins without typical export signal; [ex] proteins without typical export signal but have a known extracytoplasmic localization.

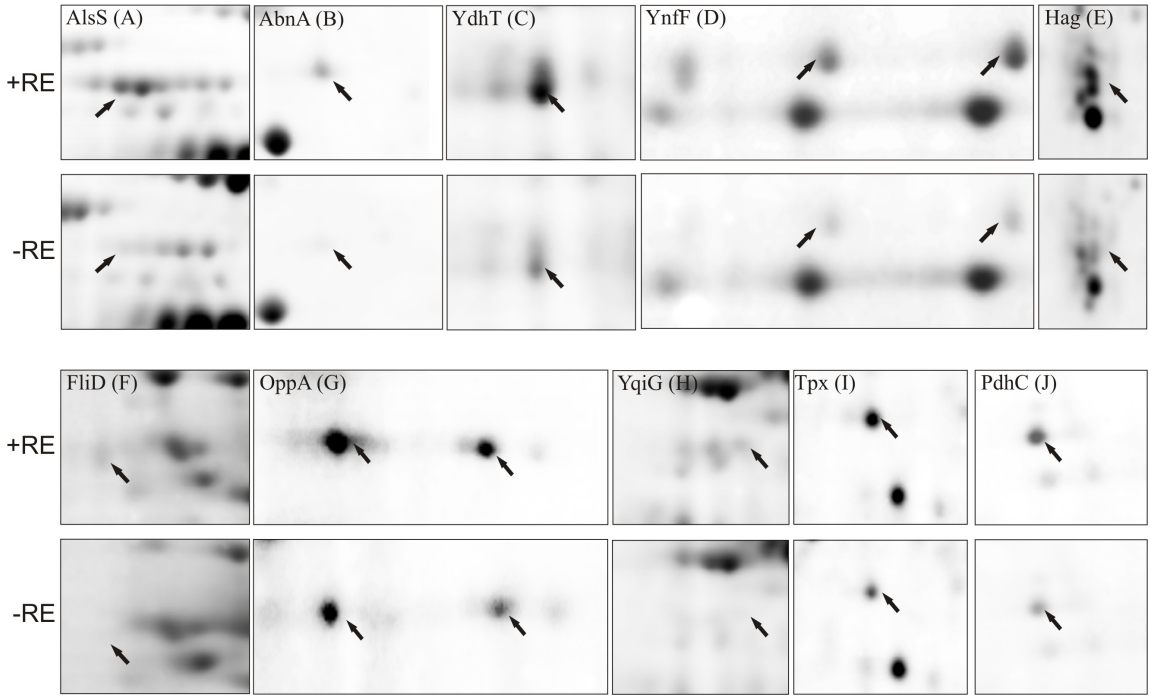

Figure 7 Bacterial proteins up-regulated by root exudates during transition phase. Taken from representative 2-D gels; +RE – presence of root exudates, - RE – absence of root exudates.

Expression of seven proteins was decreased in presence of root exudates. Only two of them, chitosanase (Csn) and endo-β-1,3-1,4 glucanase (BglS) possessed known signals for secretion (Figure 8A, B). The rest of the down-regulated proteins: PtsI, PdhC, RocA, YcgN were cytoplasmic (Figure 8C, D, E), with one involved in cell detoxification, YceE (Figure 8F).

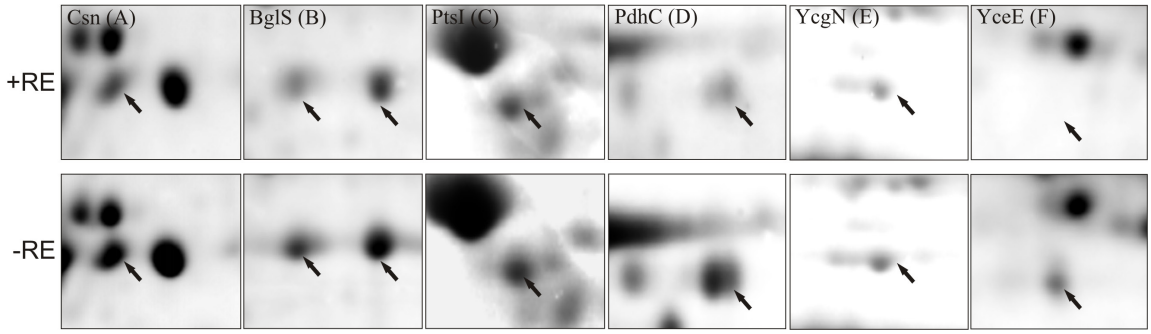

Figure 8 Bacterial proteins down-regulated by root exudates during transition phase. Taken from representative 2-D gels; +RE – presence of root exudates, - RE – absence of root exudates.

2.5.2 Differentially secreted proteins at stationary phase

Analysis of extracellular proteins isolated from cultures grown at stationary phase and separated by 2-D electrophoresis (WT+RE/WT-RE) revealed 19 proteins differentially expressed in response to root exudates (

Table 5). In contrast to the proteins obtained from the transition growth phase, a higher number of proteins (12) were down-regulated, while seven were up-regulated. When the proteins were grouped according to functional categories, the highest percentage of stimulated proteins belonged to the group involved in carbohydrate degradation, metabolism of amino acids and proteins involved in transport (Figure 9).

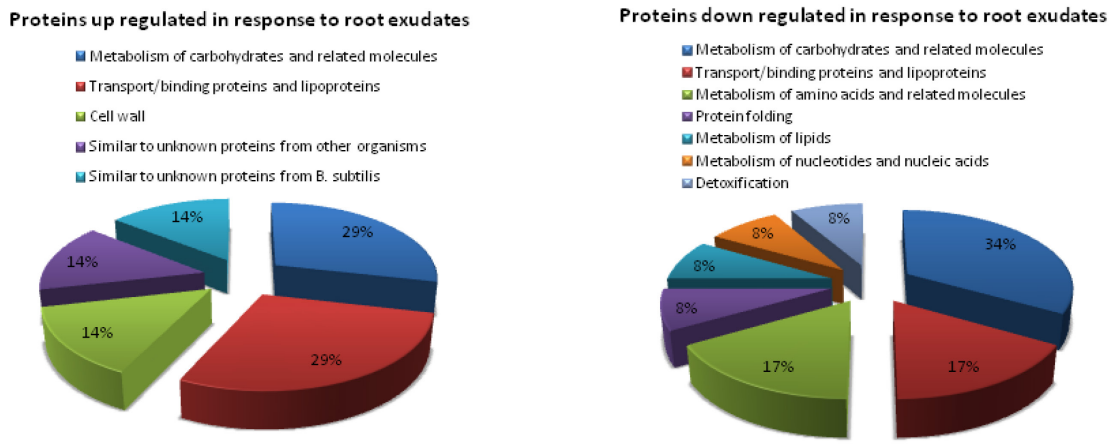

Figure 9 Contribution of functional categories to the pie chart as a percentage of differentially expressed secreted proteins isolated from cells grown until entry in to the stationary phase.

From the 19 proteins affected in their expression by root exudates, 10 possessed signals for secretion. Interestingly, the Mdh (malate dehydrogenase) protein (bearing no predicted signal for secretion, and with a clearly cytoplasmic function) was identified as being part of the extracellular secretome. Mdh was heavily expressed when root exudates were present in the medium (6.4 fold change) (Figure 10G). The strongest response to maize root exudates was observed in case of a protein involved in cell wall turnover (PonA). Although, PonA protein represents only 0.49% of the total relative spots volume, it was secreted to the growth medium only when root exudates were added (Figure 10A). In addition, a protein with putative chitin binding function (the *RBAM 1754* gene product), responded with nine times higher expression when root exudates were added into the growth medium (Figure 10B). Secre-

tion of glucomannan degrading enzyme, YdhT, was 5.75 times higher when compared to the control (Figure 10C). Increase in secretion of proteins with function in transport of phosphate (PstS, 2.9 fold change) and oligopeptides (AppA, 5.79 fold change) was also observed (Figure 10D, E, respectively). YrpD, putative lipoprotein, of yet unknown function, responded to root exudates at nearly 5 times higher secretion (Figure 10F).

Table 5 Differentially expressed proteins in response to root exudates, isolated from stationary phase.

	Protein	Function/ Similarity	Fold change	*p* value [a]	Export signal [b]
	Up-regulated				
1	PonA	penicillin-binding proteins IA/IB	10.00	0.001	Sec
2	RBAM 1754	putative chitin binding protein	9.09	<0.001	Sec
3	Mdh	malate dehydrogenase	6.43	<0.001	- [ex]
4	AppA	oligopeptide ABC transporter	5.79	0.035	Lipo
5	YdhT	β-1,4-mannanase RBAM_035930	5.75	0.002	Sec
6	YrpD	conserved hypothetical protein RBAM_010640	4.77	0.009	Sec
7	PstS	phosphate ABC transporter (binding protein)	2.90	0.009	Lipo
	Down-regulated				
8	DnaK	class I heat-shock protein (molecular chaperone)	-1.49	0.018	-
9	FeuA	iron-binding protein	-1.61	0.014	Lipo
10	Pta	phosphotransacetylase	-1.61	0.008	-
11	Asd	aspartate-semialdehyde dehydrogenase	-2.09	0.014	-
12	RocF	arginase	-2.31	0.001	-
13	Eno	enolase	-2.37	0.017	-
14	FbaA	putative translaldolase	-2.74	0.024	-
15	PenP	beta-lactamase precursor	-3.22	0.044	Sec
16	FabI	enoyl-[acyl-carrier-protein] reductase	-4.03	0.016	-
17	PtsH	phosphocarrier protein HPr component	-4.94	0.021	-
18	YfkN	putative multifunctional phosphoesterase	-5.73	0.039	RR[c]
19	YclQ	putative ferrichrome ABC transporter	-5.83	0.019	Lipo

a) Proteins were considered significantly different when p value < 0.05.

b) Export signals are Sec-type signal peptides (Sec); lipoprotein signal peptides (Lipo); twin-arginine signal peptides (RR); - proteins without typical export signal; [ex] proteins without typical export signal but have a known extracytoplasmic localization.

c) Despite the presence of RR/KR signal peptide, secretion is not Tat dependent (Antelmann *et al.* 2001).

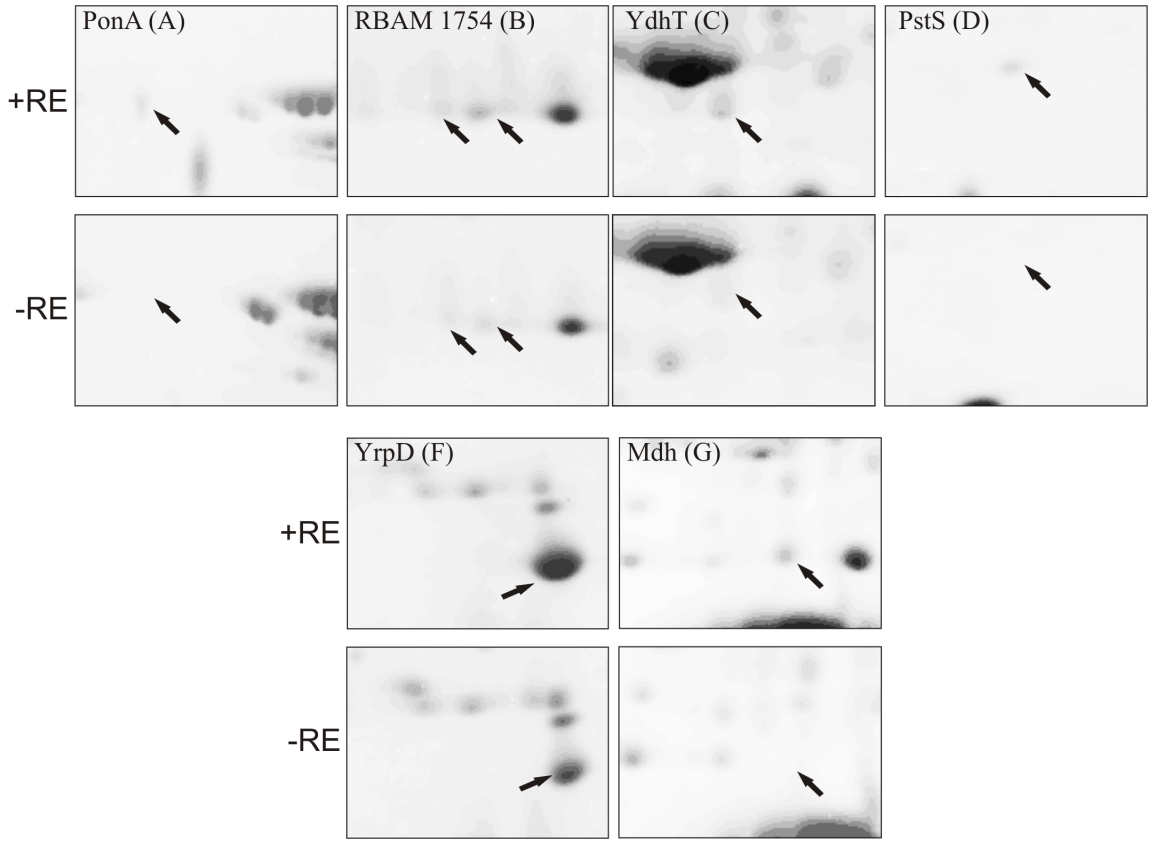

Figure 10 Bacterial proteins up-regulated by root exudates during stationary phase. Taken from the representative 2-D gels; +RE – presence of root exudates, - RE – absence of root exudates.

From the 12 proteins whose secretion decreased in the presence of root exudates, only four were found to have a secretion signals. Between them, two of the proteins involved in iron transport (FeuA, YclQ) (Figure 11A, B). Additional proteins, whose expression was decreased included YfkN (Figure 11C), which is involved in the degradation of nucleotides and nucleic acids, and PenP, the precursor of β lactamase (Figure 11D). From the eight proteins, without a typical signal peptide, DnaK, RocF and Eno were predicted by SecretomeP algorithm to be secreted via non signal peptide triggered secretion (Figure 11E, H, I). Functions of the other proteins: Pta, Asd, FbaA, (Figure 11F, G, J), FabI, PtsH (not indicated), suggests their localization in cytoplasm, and their presence in extracellular proteome is most likely due to the lysis during cells culturing.

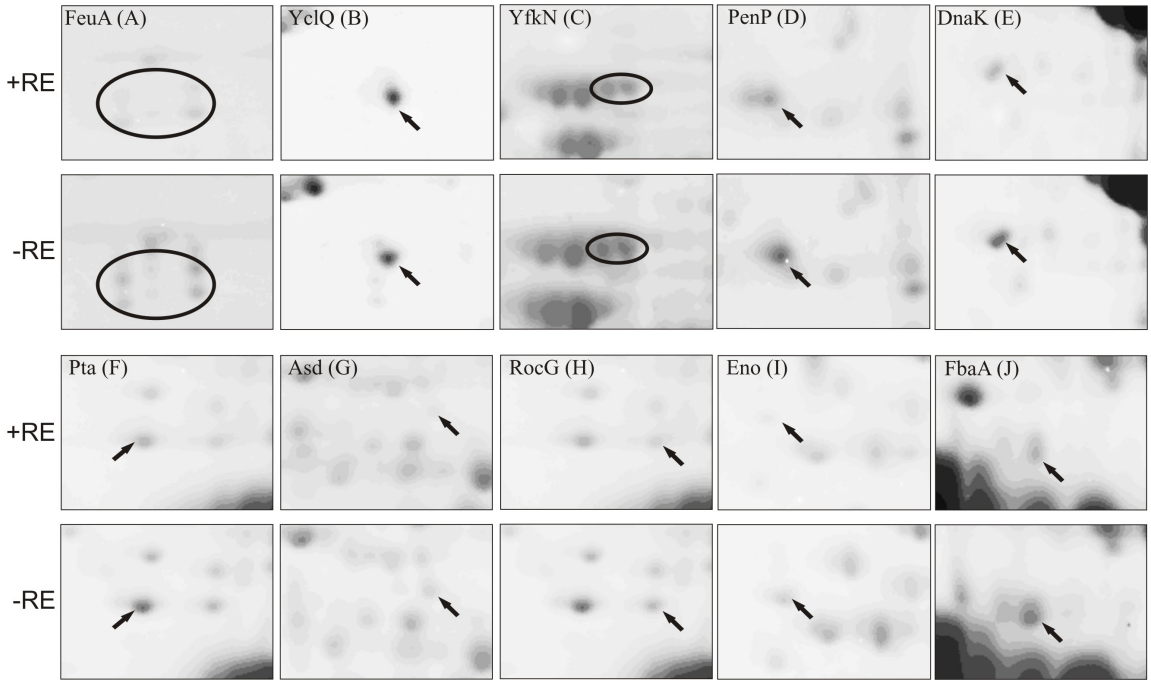

Figure 11 Bacterial proteins down-regulated by root exudates during stationary phase. Taken from representative 2-D gels; +RE – presence of root exudates, - RE – absence of root exudates.

2.6 Determination of involvement of sigma factors and global regulators in response to root exudates – extracellular proteome analysis

In order to gain more knowledge about regulatory factors involved in secretion of proteins in response to signals coming from root exudates, six FZB42 strains bearing gene knock out mutations were analyzed. We included in our analysis strains with disrupted *degU* and *abrB* genes, encoding global transcriptional regulators of gene expression during the transition to stationary phase. In addition, three strains with disrupted ECF sigma factors: *ΔsigM, ΔsigV, ΔsigX*, and the general stress response regulator, *sigB*, were also investigated. All strains were grown in presence of root exudates until transition phase, under conditions exactly the same as used in growth of wild type. Subsequently, isolated proteins were subjected to the 2-DE. Resulting gels were then compared in a way to answer the questions: i) what is the response of those mutants to root exudates?; ii) which genes are under control of alternative sigma factors or global regulators? In this case, extracellular proteomes of the mutants were compared to the extracellular proteome of wild type obtained from cultures grown in the presence of root exudates.

The expression of a protein was considered as being regulated by root exudates and directed by one of the targeted regulatory proteins, if: i) expression of a protein was altered in comparison of the mutant

strain to the wild type grown at standard conditions (fold change -1.5 ≤ ≥ 1.5, $p \leq 0.05$); ii) expression of a protein was directly regulated by one of the regulators, in case of alternative sigma factors down-regulated, and either down or up-regulated in case of global regulators (which can repress or induce the expression of genes) when compared to the wild type; iii) protein expression was influenced by root exudates in the wild type.

2.6.1 Transition state regulators

2.6.1.1 The ΔdegU strain

To obtain more information about the involvement of the DegU-DegS regulon in bacterial response to root exudates, secretome analysis was carried out. Since transcription of many extracellular *Bacillus* proteins is dependent on DegU, we expected to detect less proteins in the secretome of the mutant strain.

Out of 177 detectable protein spots, 95 different protein entries were found for the FZB42 Δ*degU* strain. When the secretome of DegU mutant was compared with the secretome of the wild type (Δ*degU*/FZB42 WT) 3 proteins were found dramatically repressed (AmyE, Bpr, Vpr), and 13 proteins were missing on the representative gel of DegU mutant. Most of them were proteases and carbohydrate degrading enzymes, probably governed by the DegU-DegS two component system (Mader *et al*. 2002). Among identified proteins, 15 were previously reported to belong to the DegU-S regulon (Mader *et al.* 2002).

In order to investigate the possible contribution of DegU in response of FZB42 to maize root exudates, two data sets were created i) extracellular proteins of the DegU mutant were compared to that of the secreted proteins of FZB42 wild type grown under same conditions in absence of root exudates, and a data set of both significantly up and down-regulated proteins was created (Δ*degU*+ root exudates /WT+ root exudates), (fold change -1.5 ≤ ≥ 1.5, $p \leq 0.05$), ii) proteins belonging to the DegU regulon differentially expressed in wild type in presence of root exudates. Subsequently, obtained lists were compared and proteins fulfilling simultaneously those conditions were considered as regulated by root exudates via involvement of DegU.

Among the 65 differentially expressed proteins, 15 were already reported as controlled by DegU-S regulon. Surprisingly, FliD, FlgK and Hag were found down regulated, which is in contrary to the previous findings indicating that DegU inhibits expression or the activity of an alternative sigma factor SigD, which is the transcriptional factor for these genes (Tokunaga *et al.* 1994, Ogura and Tanaka 1996, Ogura *et al.* 2001, Amati *et al*. 2004).

According to Figure 12, PdhC is a good example for DegU dependent positive control and root exudate seems to enhance concentration of DegU due to higher expression in their presence. The same is true for OppA and Csn which are known to be regulated by DegU. Interestingly, for one protein RBAM 1764 of unknown function, the consensus binding sequence for DegU was found. However, this protein is more expressed in the mutant strain which could indicate that DegU acts in this case as a negative regulator (Ogura *et al*. 2001). Moreover, root exudates seems to act also as a repressing factor for transcription of this gene.

Table 6 Extracellular protein responses to root exudates mediated by DegU.

		Fold change	***p*-value**	**Fold change**	***p*-value**	**Functional clssification**	**Transcriptional factor**
Gene	**Function/similarity**	**WT+/WT-**		**degU +/WT+**			
1 *pdhC*	Pyruvate dehydrogenase	1.96	0.033	-17.85	0.005	Metabolism of carbohydrates	-
2 *csn*	Chitosanase	-1.56	0.007	-2.98	0.009	Metabolism of carbohydrates	DegU, AbrB
3 *oppA*	Oligopeptide ABC transporter	2.34	0.022	-1.96	0.006	Transport/binding proteins and lipoproteins	DegU
4 *RBAM 1764*	Function unknown	-	-	10	0.009	Hypothetical protein	-

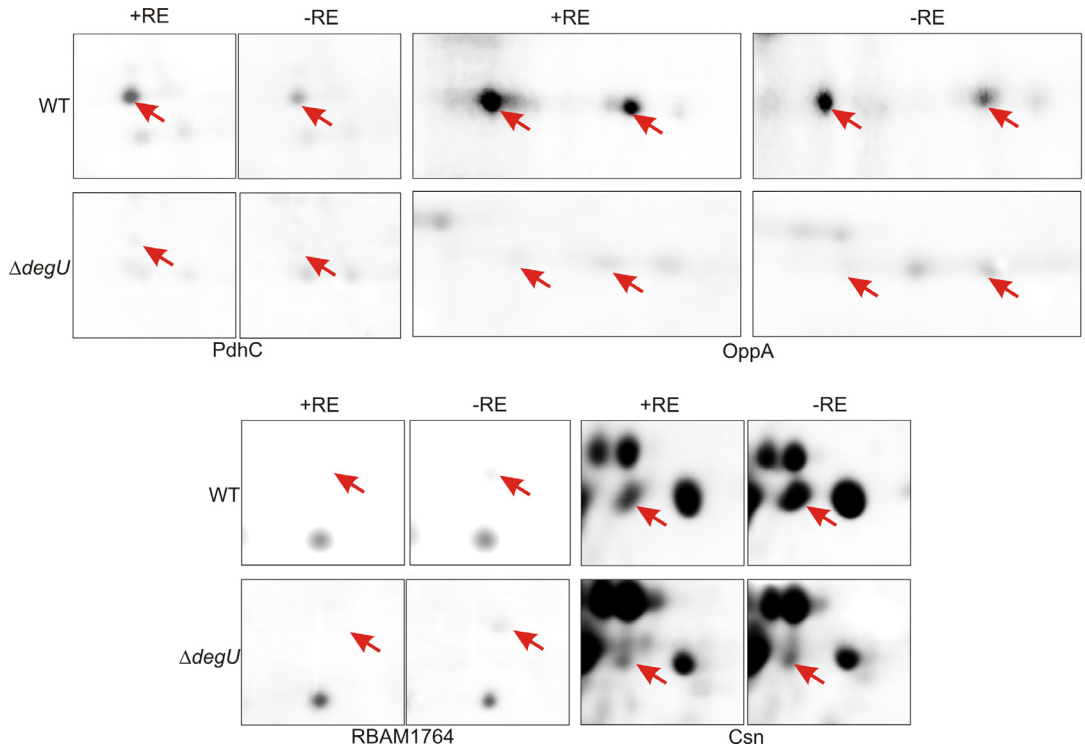

Figure 12 Proteins (indicated by arrows) expressed in a DegU-dependent manner. The images are representative sections of 2-DE gel profiles of proteins extracted from transition growth phase of cells of *B. amyloliquefaciens* FZB42 (WT) strain and the Δ*degU* mutant grown in the presence (+RE) or in the absence (-RE) of root exudates.

2.6.1.2 *The ΔabrB strain*

AbrB acts mainly as a negative regulator of extracellular proteins. However, there are several examples for positive regulation of extracellular proteins by AbrB: AmyE and YdhT (Strauch 1995; Chumsakul *et al*. 2010).

Computer assisted analysis of FZB42Δ*abrB* secretome resulted in detection of approximately 113 protein spots, representing 57 different protein entries. To determine the possible role of AbrB in response to root exudates FZB42 mutant strain with disrupted *abrB* gene was analyzed in the same way as FZB42 Δ*degU*. In this way 32 proteins have been significantly differentially expressed (fold change -1.5 ≤ ≥ 1.5, $p \leq 0.05$) (Δ*abrB*/FZB42 WT), 10 of which were previously reported as being AbrB dependent (Strauch *et al* 1989; Hamon *et al*. 2004), and 14 were missing from the representative gel of AbrB mutant. Involvement of AbrB in gene repression during the response to root exudates was clear in case of YnfF (Figure 13), Csn and transcription activation was evident for the YdhT. However, effect of AbrB on those genes is already known (Rivas *et al*. 2000, St John *et al*. 2006, Sadaie *et al*. 2008).

Taking under consideration fact that AbrB can act both as repressor and as a activator, we were looking for proteins which are either more expressed or significantly reduced in the mutant strain. According to Figure 13 AbnA is a good example of AbrB dependent positive control, and root exudates seems to enhance concentration of AbrB due to higher expression in their presence. However, it has been previously reported that in *B. subtilis* transcription of its gene product is regulated by other transcriptional repressor (Franco *et al*. 2007)

Table 7 Extracellular protein responses to root exudates mediated by AbrB.

		Fold change	***p*-value**	**Fold change**	***p*-value**	**Functional classification**	**Transcriptional factor**
Gene	**Function/similarity**	**WT+/WT-**		**abrB +/WT+**			
1 *abnA*	Arabinan-endo 1,5-alpha-L-arabinase	3.9	0.001	-10.6	0.001	Metabolism of carbohydrates	-
2 *ynfF*	Endo-xylanase	3.14	0.030	6.01	0.004	Metabolism of carbohydrates	AbrB

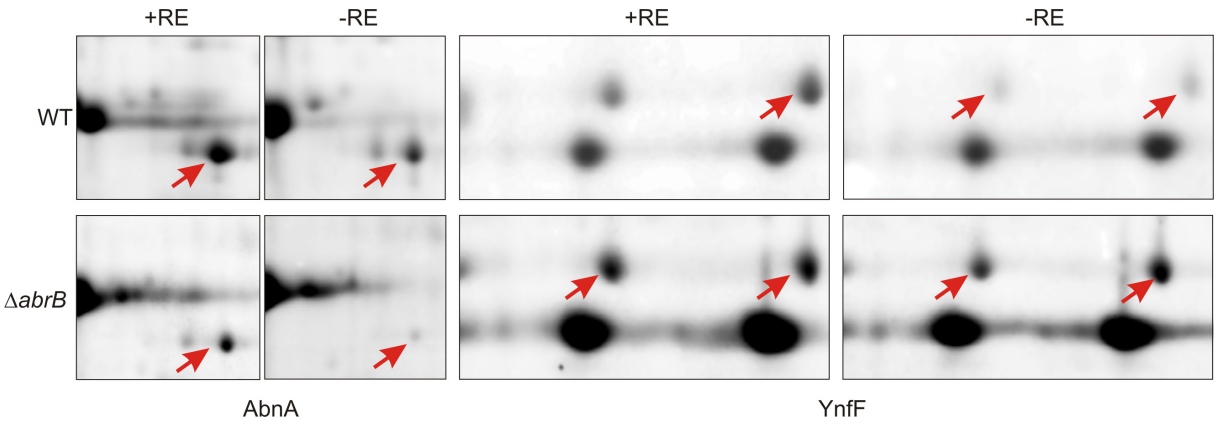

Figure 13 Proteins (indicated by arrows) expressed in a AbrB-dependent manner. The images are representative sections of 2-DE gel profiles of proteins extracted from transition growth phase of cells of *B. amyloliquefaciens* FZB42 (WT) strain and the Δ*abrB* mutant grown in the presence (+RE) or in the absence (-RE) of root exudates.

2.6.2 Heat shock sigma factor ΔsigB

In order to gain knowledge about the participation of the alternative SigB sigma factor in response of FZB42 to root exudates appropriate comparisons were performed. First, proteins under control of SigB were determined by comparison of Δ*sigB*/FZB42 WT proteomes, resulting in 34 proteins among 167 identified protein spots. Due to the nature of sigma factors, only proteins that were down-regulated were considered as controlled by SigB. Subsequently, similarly to the comparison made in the case of transition state regulators, the obtained data set was compared with the proteins whose expression was not altered by root exudates in the FZB42Δ*sigB* proteome (Δ*sigB*+RE/Δ*sigB*-RE) and with record of FZB42 wild type proteins affected by root exudates (WT+RE/WT-RE) listed in Table 4. However, none of the proteins matched those criterions, suggesting that SigB is not involved in the response of extracellular proteins to root exudates in FZB42. This corroborated previous microarray studies showing minor involvement of SigB in response to root exudates (Fan, 2011).

2.6.3 ECF sigma factors SigM, SigV and SigX

The extracytoplasmic function sigma factors, SigM, SigV and Sig X, were analyzed in order to find out their possible role in cellular responses to root exudates. Analysis was done as described for SigB. Only one protein, a β-1,4-mannanase (YdhT), an enzyme taking part in extracellular digestion of glucomannan was found to be involved in response to root exudates via contribution of the ECF SigM sigma factor (Table 8, Figure 14). YdhT was identified as being under control of another ECF, SigX (Table 9, Figure 15), as well, suggesting that those sigma factors are overlapping in their regulatory circuits.

Table 8 Extracellular protein responses to root exudates mediated by SigM.

		Fold change	***p*-value**	**Fold change**	***p*-value**	**Functional classification**	**Transcriptional factor**
Gene	**Function/similarity**	**WT+/WT-**		**sigM+/WT+**			
1 *ydhT*	β-1,4-mannanase	1.62	0.036	-3.54	0.00	Metabolism of carbohydrates	-

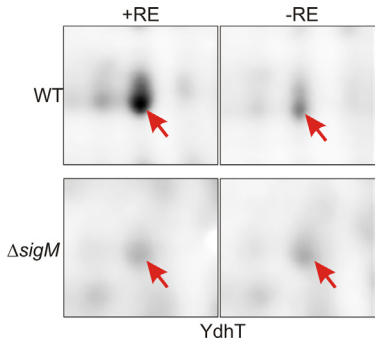

Figure 14 Proteins (indicated by arrows) expressed in a SigM-dependent manner. The images are representative sections of 2-DE gel profiles of proteins extracted from transition growth phase of cells of *B. amyloliquefaciens* FZB42 (WT) strain and the Δ*sigM* mutant grown in the presence (+RE) or in the absence (-RE) of root exudates.

Table 9 Extracellular protein responses to root exudates mediated by SigX.

		Fold change	***p*-value**	**Fold change**	***p*-value**	**Functional classification**	**Transcriptional factor**
Gene	**Function/similarity**	**WT+/WT-**		**sigX+/WT+**			
1 *ydhT*	β -1,4-mannanase	1.62	0.036	-2.48	0.002	Metabolism of carbohydrates	-

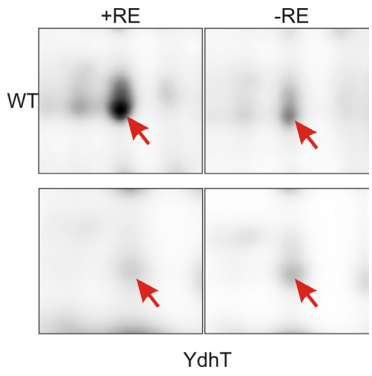

Figure 15 Proteins (indicated by arrows) expressed in a SigX-dependent manner. The images are representative sections of 2-DE gel profiles of proteins extracted from transition growth phase of *B. amyloliquefaciens* FZB42 (WT) strain and the Δ*sigX* mutant grown in the presence (+RE) or in the absence (-RE) of root exudates.

According to the Figure 14 and Figure 15, YdhT is an example of SigM and SigX controlled expression. Furthermore, it seems that effect of alternative sigma factors is enhanced in presence of root exudates.

None of the proteins has been identified as responsive to root exudates via involvement of SigV. Thus it can be hypothesized, that contribution of SigV in bacterial response to root exudates is very weak, if any. Similar results gave microarray analysis of FZB42, where only four genes were proposed to be regulated by root exudates via the involvement of SigV (Fan, 2011).

2.7 Two dimensional map of cytosolic proteins of *B. amyloliquefaciens* FZB42

2.7.1 Map of the theoretical proteome of *B. amyloliquefaciens*

To simulate distribution of all predicted proteins, the reference map according to the genome sequence was prepared. A synthetic proteome gel was created by using the software JVirGel (www.jvirgel.de), (Figure 16). As with *B. subtilis* 168 (Buttner *et al*. 2001), proteins of a virtual gel were distributed in two regions: 1) alkaline – neutral and 2) basic. Of the total number of predicted ORFs (3693), almost two thirds of the proteins are found across p*I* ranges from 3 to 7.5 (Figure 16). The remaining third of alkaline proteins are located in the p*I* range from 8 to 13. In addition, most of the predicted membrane proteins with more than four spanning domains, are distributed in the basic region (prediction was made using the TMHMM software).

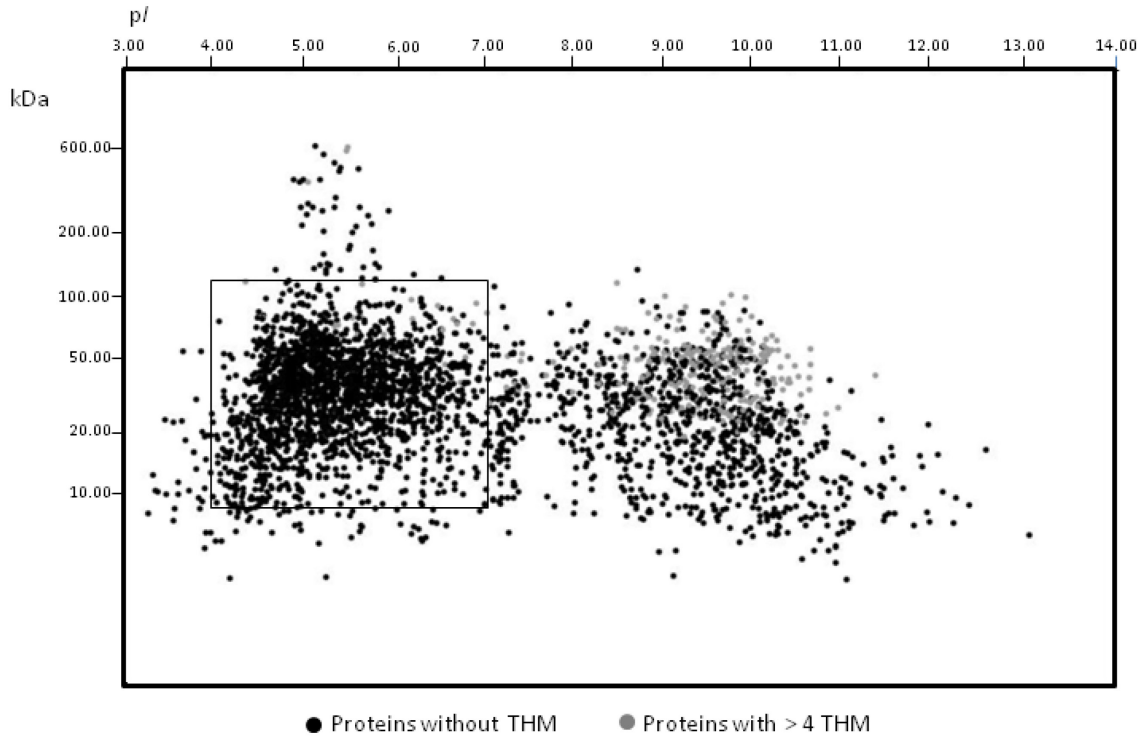

Figure 16 Theoretical proteome of *B. amyloliquefaciens.* The figure represents 2-D gel separation of total proteome according to calculated isoelectric point (p*I*) and molecular weight (MW). The rectangle indicates the analytical window of the 2-DE.

2.7.2 Reference map of cytosolic proteins of cells grown in standard medium at transition phase

As most proteins were distributed in the acidic – neutral range, IPG strips of pH 4-7 were applied to separate the cytoplasmic proteins of *B. amyloliquefaciens* in first dimension.

FZB 42 cells were cultivated aerobically until the late exponential or transition phase (Figure 1) and then disrupted using a French press. The soluble proteins (500µg) were loaded onto commercially available IPG strips, and subsequently subjected to the 2-DE. The colloidal Coomassie stained gels resolved approximately 788 distinct spots. After in-gel trypsin digestion, MALDI-TOF-MS and/or ESI-MS, 706 spots were successfully identified, this corresponds to an identification rate of approximately 90%. A total of 461 different proteins were identified, ranging in molecular mass from 7 to 140 kD, and a p*I* range lying between 3.9 and 10.3, were identified (Figure 17). This covers around 12.4% of the theoretical proteome of FZB42 (Appendix Table 3). Identified protein spots represented approximately 33% of the predicted proteome of *B. amyloliquefaciens* within the chosen pH range. It is likely that the rest of the theoretical proteome was not detected because many proteins: 1) were present at low abundance, 2) were poorly solubilised in the solvents used for extraction, or 3) were not synthesized under the given conditions. The majority of the 461 identified proteins (93%, total of 430) were assigned as cytoplasmic proteins. The other proteins were predicted as extracytoplasmic, with cleavage sites for signal peptidase I (eight proteins) and signal peptidase II (two proteins). One protein was predicted to be secreted via the Tat pathway, and 20 proteins were secreted via non classical pathways. The presence of extracellular proteins may be explained by co-purification with those from the periplasmic space.

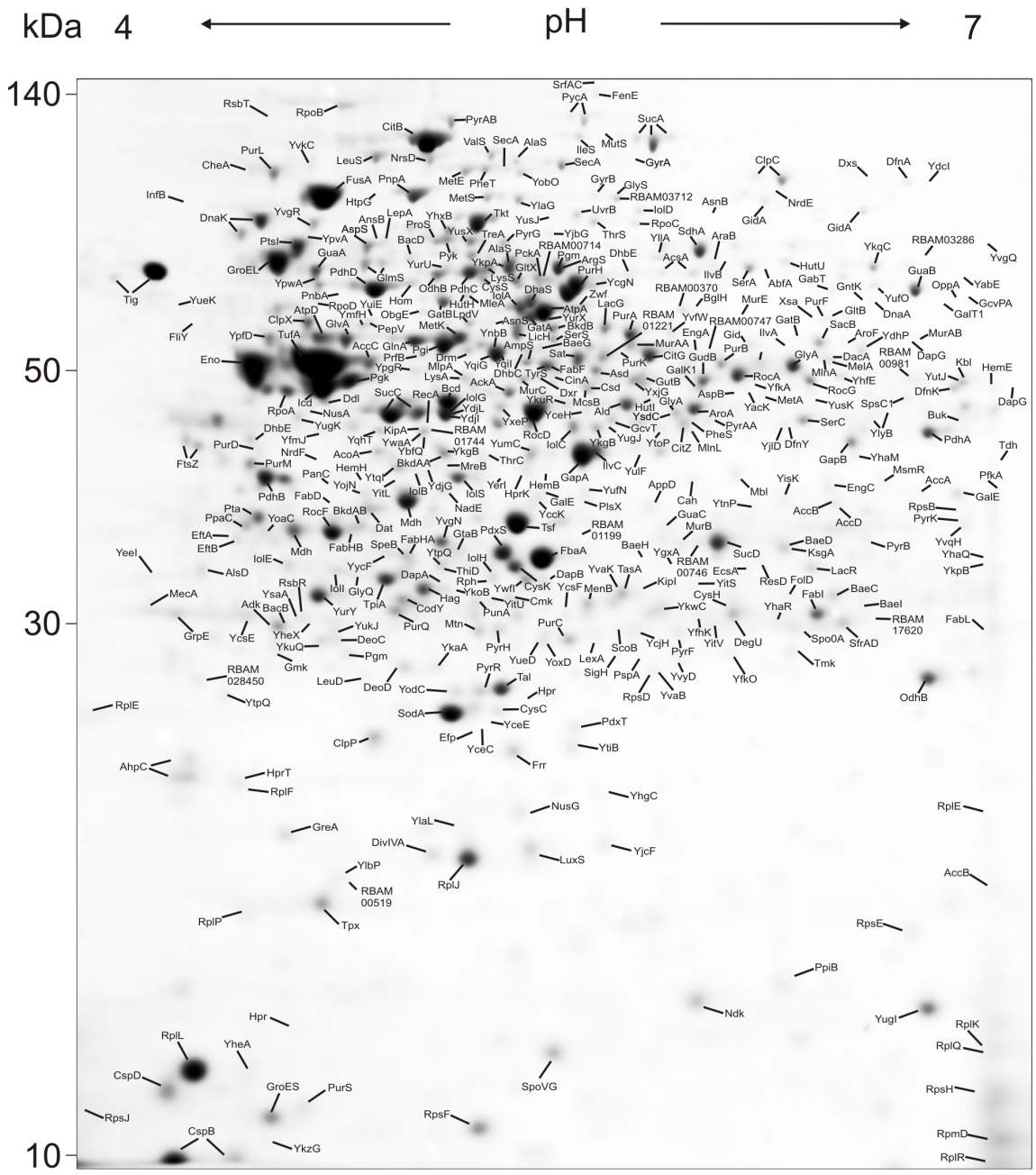

Figure 17 Reference 2-D electrophoresis map of cytosolic *B. amyloliquefaciens* FZB42 proteins. Cells were grown in 1C medium and were harvested during transition phase.

2.7.2.1 Functional classification and localization of the identified proteins

The 461 identified proteins of *B. amyloliquefaciens* were grouped into 22 functional categories based on the Cluster of Orthologous Groups of protein classification scheme. The largest group of identified proteins was assigned to the class of proteins involved in amino acid transport and metabolism (COG category E: 14%, 64 proteins). Other COG categories that were well represented by the identified proteins were 1) the group involved in translation (COG category J: 11%, 51 proteins), 2) the group in-

volved in energy production and conversion (COG category C: 10%, 48 proteins), and 3) the group involved in sugar transport and metabolism-related proteins (COG category G: 9%, 40 proteins) (Figure 18A).

The function of just 14% of the identified proteins was unknown (category S 3%, R 9%, X 2%). Considering that almost one third of the predicted ORFs consisted of unknown proteins, experimental underrepresentation of that group in our analysis may indicate that the majority of those proteins are involved in functions unique to *B. amyloliquefaciens* and are not required under the conditions chosen for growth of FZB42 in this study.

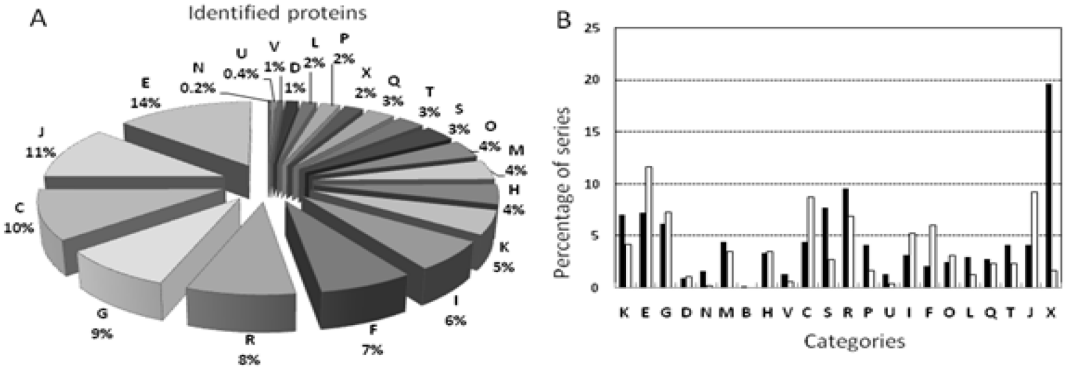

Figure 18 Categorization of the *B. amyloliquefaciens* proteins according to the COG categories. (A) Functional distribution of the identified proteins. (B) Schematic presentation of the COG categories of theoretical (black bars) and identified proteins (open bars). K, transcription; E, amino acid transport and metabolism; G, carbohydrate transport and metabolism; D, cell cycle control, mitosis and meiosis; N, cell motility; M, cell wall/membrane biogenesis; B, chromatin structure and dynamics; H, coenzyme transport and metabolism; V, defense mechanisms; C, energy production and conversion; S, function unknown; R, general function prediction only; P, inorganic ion transport and metabolism; U, intracellular trafficking and secretion; I, lipid transport and metabolism; F, nucleotide transport and metabolism; O, posttranslational modification, protein turnover, chaperones; L, replication, recombination and repair; Q, secondary metabolites biosynthesis, transport and catabolism; T, signal transduction mechanisms; J, translation; X, not in Clusters of Orthologous Groups.

2.7.2.2 *Codon adaptation index*

Synonymous codons are not always used at the same frequency in a given genome. Highly expressed proteins are encoded by genes containing a high percentage of codons which are efficiently recognized by the most abundant tRNAs (Ikemura 1985). The calculation of the Codon Adaptation Index (CAI) for a gene allows measurement of the codon usage bias and thus the propensity of the corresponding protein to be highly synthesized (Sharp and Li 1987). The CAI of all 3693 ORFs of *B.*

amyloliquefaciens was calculated. A value of 1.0 indicates maximum codon usage fit, and values less than 1.0 indicate use of less preferred codons. Figure 19 shows a comparison of the CAI distribution of identified proteins at 4-7 pH range to all predicted proteins. The proteins encoded by genes with a CAI value above 0.5 accounts for 83% of the predicted proteome. Almost 96% of the 461 identified proteins have CAI value above 0.5, however not all proteins with CAI values above 0.8 were identified.

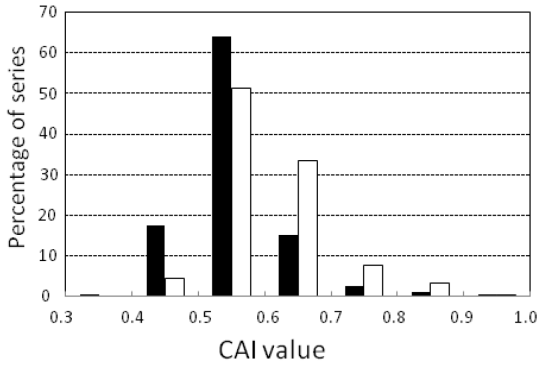

Figure 19 Frequency distribution of the CAI values of 3693 ORFs of *B. amyloliquefaciens* FZB42 (black bars) and of 461 genes encoding proteins identified on 2-D gel pH 4–7 (open bars).

2.7.2.3 *Grand average of hydropathy*

Proteins with highly hydrophobic regions are difficult to detect under standard 2-DE conditions. The GRAVY (Grand Average of Hydropathy) index describes the hydropathy of a protein. The hydrophobicity of all predicted ORFs was calculated. The GRAVY value of all identified proteins ranged from -1.0 to 0.3, indicating that highly hydrophobic proteins may be lost during preparation of the proteins or during 2-DE gel electrophoresis and were therefore ignored. Thirty of all identified proteins have a GRAVY score above zero. A comparison between all predicted proteins and identified proteins shows that highly hydrophobic proteins are not present among the identified proteins. This is likely to be due to the poor solubility of these proteins in the buffers used (Figure 20). Similarly, none of the highly hydrophilic proteins were detected, despite the fact that they are likely to be easily dissolved in the buffers. A plausible explanations for this could be low cellular abundance of such proteins in the protein extract from *B. amyloliquefaciens*.

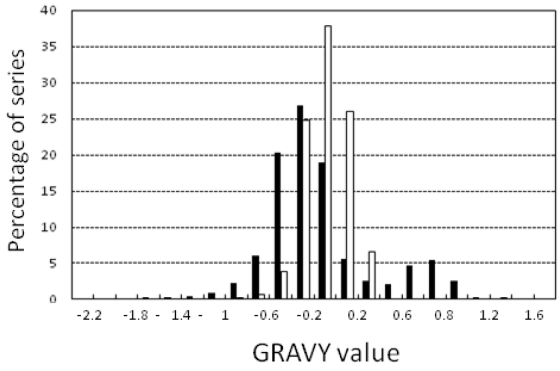

Figure 20 Frequency distribution of the GRAVY values of the 3693 ORFs of *B. amyloliquefaciens* FZB42 (black bars) and of 461 genes encoding proteins identified on 2-D gel pH 4–7 (open bars).

2.7.3 Reference map of cytosolic proteins of cells grown in minimal medium at transition phase

To check functional diversity of *B. amyloliquefaciens* the cells were cultivated in minimal medium until transition phase was reached. Coomassie stained gels revealed approximately 349 spots. Using mass spectrometry, about 245 protein entries were identified; however 25 spots could not be identified (Appendix Table 3). Interestingly, 49 proteins were exclusively expressed by the cells grown in the minimal medium (Appendix Table 4, Figure 21). Not surprisingly, the CAI index calculated for genes encoded for the newly identified proteins was below 0.55, indicating that those proteins are not preferentially expressed and their occurrence in the cell is relatively low.

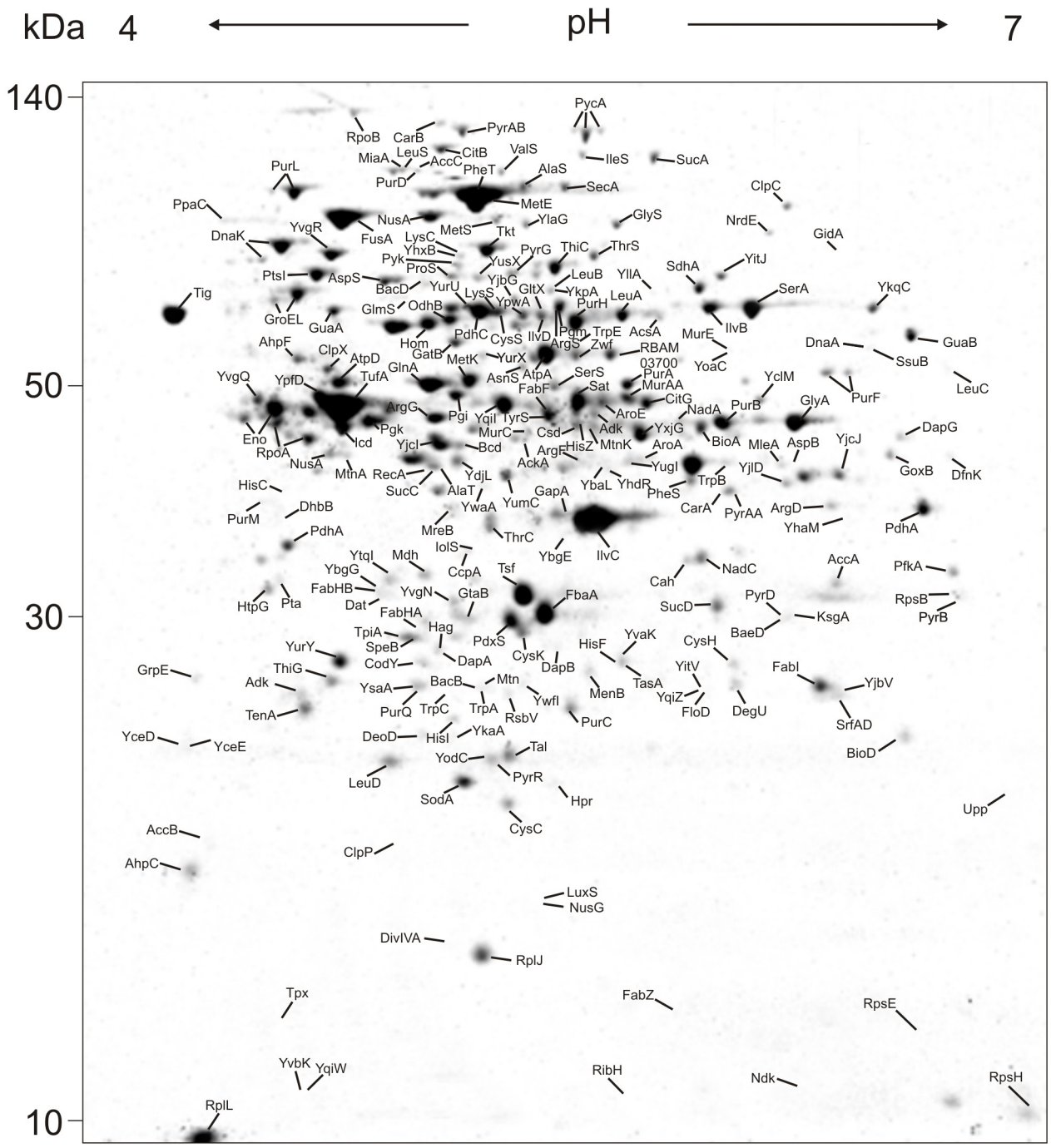

Figure 21 Cytosolic map of *B. amyloliquefaciens* FZB42 proteins. Cells were grown in minimal medium until transition phase.

2.7.4 Metabolic pathways of the *B. amyloliquefaciens* FZB42

Establishment of the cytosolic reference map of *B. amyloliquefaciens* FZB42 allowed us to reconstruct basic metabolic pathways (Figure 23).

2.7.4.1 Glycolysis

Nine enzymes catalyzing conversion of glucose to pyruvate under the tested growth conditions (protein extracts obtained from cells grown in 1C and minimal media) were identified: glucose-6-phosphate isomerase (Pgi), 6-phosphofructokinase (PfkA), fructose-1,6-bisphosphate aldolase (FbaA), triose phosphate isomerase (TpiA), glyceraldehyde-3-phosphate dehydrogenase (GapA), phosphoglycerate kinase (Pgk), phosphohexomutase (Pgm), enolase (Eno), pyruvate kinase (Pyk). The only enzyme that was not detected was glucokinase (GlcK). The CAI index of the identified glycolytic enzymes was above 0.6, while the CAI value for the glucokinase was 0.5.

2.7.4.2 Nucleotide metabolism

The proteome map consisted of 20 enzymes involved in the metabolism of purines (11) and pyrimidines (9). With the exception of phosphoribosyl glycinamide formyltransferase (PurN) and phosphoribosyl aminoimidazole carboxylase (PurE), the other 11 proteins necessary for *de novo* synthesis of purines were detected (PurABCDFHKLMSQ). Similarly, enzymes involved in *de novo* synthesis of pyrimidine ribonucleotides (PyrAA, PyrAB, PyrBCFGH, Cmk, PnpA) were almost covered by the reference map of cytosolic proteins. The proteins, orotate phosphoribosyl transferase (PyrE) and dihydroorotate dehydrogenase, class 1B (PyrD) were not detected. This may be because their expression level was under the level of detection. Only several enzymes of the pyrimidine deoxyribonucleotides *de novo* synthesis pathway were detectable.

2.7.4.3 Peptidoglycan precursor assembly

The presented proteome map contains enzymes implicating the synthesis of peptidoglycan precursor (UDP-N-acetylmuramoyl-pentapeptide) in the cytoplasm. Two consecutive enzymatic steps, catalyzed by products of *murAA/*or *murAB* and *murB* genes, are controling the conversion of UDP-D-glucosoamine to UDP-*N*-acetylmuramate. Subsequently, MurC adds L-alanine in to the peptidoglycan

precursor chain. The enzymes, catalyzing the addition of the *N*-glutamate (MurD) and *N*-alanine (MurF) dipeptides were not present in the reference map.

2.7.4.4 *Secondary metabolites synthesis*

B. amyloliquefaciens FZB42 produces secondary metabolites with antimicrobial activity (Chen *et al.* 2007). Some enzymes involved in the synthesis of bacillaene (BaeCDGHIMR) and difficidine (DfnADGKY) were detected in the proteome of cells grown in 1C medium as well as in minimal medium. In addition, several enzymes involved in bacilysin, macrolactin, fengycin and surfactin synthesis were identified.

2.7.4.5 *Stress adaptation proteins*

As with all soil bacteria, *B. amyloliquefaciens* is often subjected to unfavorable conditions within the natural environment and has a genetic mechanisms to cope with these adverse conditions. In the present study a plethora of enzymes involved in environmental adaptation were detected. These included manganese dependent superoxide dismutase (SodA), and two alkyl hydroperoxide reductases (AhpCF). The genes *yceCDEH* were probably coding for tellurium resistance proteins. The *yce* operon comprised five other genes (*yceABFGI*), which were not detected. The explanation for this could be low values of CAI index: 0.47, 0.47, 0.55, 0.45, 0.48, respectively, compared to the value higher than 0.57 for the detected proteins. Presence of the heat stress response-related ATPases ClpC and ClpX or the peptidase ClpP in the cell is crucial for tolerance to many forms of stress (Kruger *et al*. 2000). Molecular chaperones are involved in quality control (DnaK), folding (Tig) and re-folding (GroEL and GroES) of translated proteins.

2.7.5 Differences in expression of proteins caused by growth of *B. amyloliquefaciens* FZB42 in different media at transition growth phase

Although, the majority of proteins were expressed by *B. amyloliquefaciens* FZB42 grown in either 1C or minimal medium, differences in cell metabolism were observed (Figure 22). This may reflect the potential of FZB42 to adjust its metabolism to varying abiotic conditions such as substrates availability and composition (Preston-Mafham *et al*. 2002).

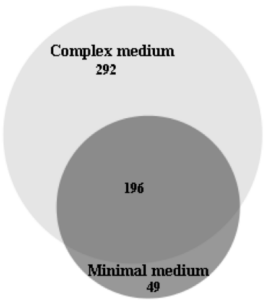

Figure 22 Venn diagram showing the number of expressed proteins in 1C and minimal media.

2.7.5.1 *Carbohydrate degradation*

One of natural habitats for *B. amyloliquefaciens* is the plant rhizosphere. To access plant derived carbon-containing substances, bacteria must effectively colonize plant roots and therefore compete with other microorganisms (Dennis *et al*. 2010) and most importantly must be able to utilize those substances. When cells of FZB42 were grown in the 1C medium supplemented with soil extract and/or root exudates, enzymes involved in their decomposition were synthesized. For example two arabinofuranosidases (AbfA and Xsa) and arabinan-endo 1.5-alpha-L-arabinase (AbnA), enzymes degrading arabinan were detected in the proteome of *B. amyloliquefaciens* grown in the 1C medium. Enzymes involved in catabolism of various plant originated carbohydrates were detected as lichenin utilization protein (LicH), and glucomannan hydrolyzing enzyme (YdhP). Moreover, proteins utilizing melibiose (MelA), trehalose (TreA), maltose (GlvA), were detected on the reference protein map. With exception of phosphoglucomutase, the remaining enzymes of the galactose degradation pathway (Leloir Pathway) were identified: galactokinase (GalK1), UDP-glucose 4-epimerase (GalE1) and galactose-1-phosphate uridyltransferase (GalT1). Three proteins, acetolactate decarboxylase (AlsD), acetolactate synthase (AlsS) and acetoin/ butanediol dehydrogenase (YjdL), involved in overflow metabolism and possibly in the synthesis of volatile compounds were also detected. Proteins involved in the degradation of *myo*-inositol (IolBCDEG) to acetyl CoA (IolA) or to glyceraldehyd (TpiA) were identified. None of the proteins involved in the utilization of plant derived compounds were detected on the 2-D gels obtained from cells grown in minimal medium.

2.7.5.2 *Amino acid metabolism*

Many proteins involved in amino acid metabolism were differentially expressed. When cells were grown in 1C medium supplemented with soil extract and/or root exudates, biosynthesis pathways for leucine, valine, isoleucine, cysteine, phenylalanine, tyrosine, methionine, lysine were induced as was a

protein involved in the conversion of proline to glutamate (YcgN). This may indicate that these amino acids were deficient in the growth media.

Enzymes for *de novo* synthesis of several amino acids were expressed exclusively by the cells of *B. amyloliquefaciens* grown in synthetic medium. Four enzymes involved in biosynthesis of tryptophan were identified: anthranilate synthase component I (TrpE), indol-3-glycerol phosphate synthase (TrpC), tryptophan synthase (alpha subunit) (TrpA), tryptophan synthase (beta subunit) (TrpB). In addition, enzymes of the arginine biosynthesis pathway were only identified in the reference proteome map of cells grown in minimal medium. Five enzymes (ArgDFG and CarAB) out of nine predicted were detected.

2.7.5.3 *Hypothetical and conserved hypothetical proteins*

51 proteins of unknown function were identified when cells were grown in minimal medium.

2.7.5.4 *Coenzymes and prostetic groups metabolism*

Enzymes involved in biosynthesis of vitamins as biotin, thiamine and riboflavin were detected only when cells were grown in minimal medium.

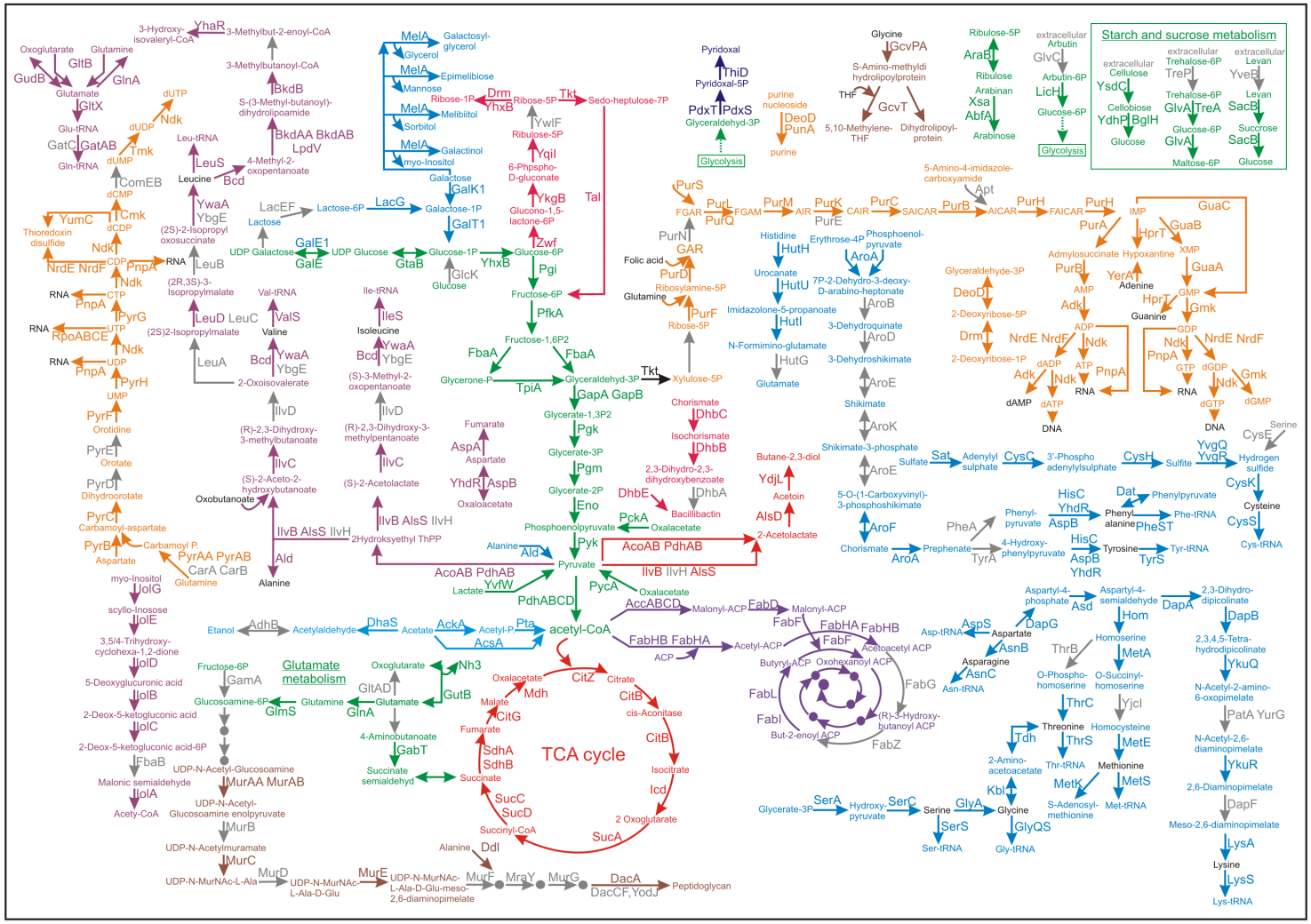

Figure 23 Assignment of identified proteins in the 2-D gel electrophoresis. Proteins represent different branches of cellular metabolism, proteins that have not been indentified thus far are shaded grey.

2.8 Differentially expressed cytosolic proteins of *B. amyloliquefaciens* in response to root exudates

To better understand how the presence of root exudates influence the cytosolic proteome of *B. amyloliquefaciens* FZB42, cells of the strain were grown in the presence and absence of root exudates. Protein extracts obtained from cells grown until the transition phase were subjected to 2-DE. Out of 461 proteins identified from wild type cells, the expression level of 21 proteins was significantly altered by supplementing root exudates. The expression level of 16 proteins was significantly up-regulated in response to root exudates, while only five proteins were repressed in their presence. A similar ratio of up and down-regulated genes was also observed in case of FZB42 transcriptional profiling (Fan 2011). Not surprisingly, based on functional classification, the majority (67%) of the up-regulated proteins were involved in carbohydrate degradation processes (Figure 24).

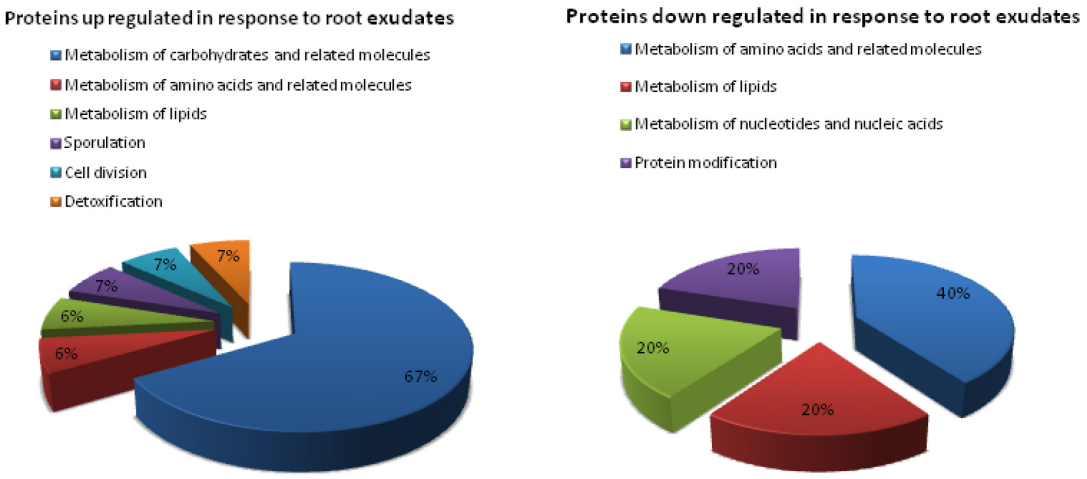

Figure 24 Contribution of functional categories to the pie chart as a percentage of differentially expressed cytosolic proteins isolated from the cells grown at transition phase.

The protein, which expression increased the most in presence of root exudates (5.7 fold change) was L-ribulokinase (AraB), an enzyme involved in utilization of arabinose. Among the proteins induced by root exudates were also two arabinofuranosidases: AbfA and Xsa, esterases that hydrolyze the terminal non-reducing α-L-arabinofuranoside residues in α-L-arabinosides, one of the main constituents of plant cell walls (http://www.cazy.org/). The presence of root exudates led to a significant increase in the level of the enzyme involved in decomposition of glucomannan (YdhP). Three enzymes involved in the hydrolysis of maltose (GlvA), melibiose (MelA) and sucrose (SacB) were found at greater level in cultures where root exudates were added. In addition, two proteins involved in catabolism of inosi-

tol were up-regulated: IolC, an enzyme forming 2-deoxy-5-keto-gluconic acid-6-phosphate and IolH, an enzyme whose function in inositol metabolism is not well understood (Yoshida *et al*. 2008). Only one protein was assigned to the category of proteins involved in metabolism of amino acids, 1-pyrroline-5-carboxylate dehydrogenase (YcgN). This enzyme converts proline to glutamate, and is particularly interesting, since a gene of identical function in Gram-negative *Pseudomonas putida* KT2442 was strongly induced by the presence of root exudates (Vilchez *et al.* 2000). Increased expression was observed for two enzymes involved in cell envelope and cellular processes: cell-division initiation protein (FtsZ) and spore coat polysaccharide biosynthesis protein (SpsC1), (Table 10, Figure 25).

Among proteins repressed by the presence of root exudates were, putative branched-chain-amino-acid aminotransferase (YwaA) and hypothetical aminotransferase encoded by the gene RBAM003700. Also, a protein involved in the synthesis of fatty acids (AccC), purines (PurH) and the HPr kinase (HprK) was down-regulated in presence of root exudates (Table 10, Figure 25).

Table 10 Differentially expressed proteins of *B. amyloliquefaciens* FZB42 during growth in presence of root exudates compared to the cells grown without their supplementation.

	Gene	Protein function/ similarity	Fold change[a]	*p*-value	Functional classification
Up-regulated					
1	*araB*	ribulokinase	5.70	0.011	Metabolism of carbohydrates
2	*xsa*	alpha-N-arabinofuranosidase II	2.63	0.013	Metabolism of carbohydrates
3	*ypgR*	conserved hypothetical protein	2.57	0.015	Similar to unknown proteins from *B. subtilis*
4	*abfA*	alpha-L-arabinofuranosidase I	2.54	0.045	Metabolism of carbohydrates
5	*yhaR*	enoyl-CoA hydratase	2.02	0.034	Metabolism of lipids
6	*spsC1*	spore coat polysaccharide biosynthesis protein	1.87	0.029	Cell envelope
7	*glvA*	maltose-6'-phosphate glucosidase	1.87	0.000	Metabolism of carbohydrates
8	*iolC*	inositol utilization protein C	1.84	0.010	Metabolism of carbohydrates
9	*ycgN*	1-pyrroline-5-carboxylate dehydrogenase	1.78	0.011	Metabolism of amino acids
10	*ydhP*	putative beta-glucosidase	1.66	0.003	Metabolism of carbohydrates

11	*iolH*	inositol utilization protein H	1.64	0.004	Metabolism of carbohydrates
12	*ahpF*	alkyl hydroperoxide reductase	1.59	0.011	Detoxification
13	*melA*	alpha-D-galactoside galactohydrolase	1.57	0.015	Metabolism of carbohydrates
14	*sacB*	levansucrase	1.55	0.013	Metabolism of carbohydrates
15	*odhB*	dihydrolipoamide acetyltransferase	1.53	0.010	Metabolism of carbohydrates
16	*ftsZ*	cell division protein	1.51	0.023	Cell division
Down-regulated					
17	*ywaA*	branched-chain amino acid aminotransferase	-1.55	0.020	Metabolism of amino acids
18	*hprK*	HPr kinase/phosphorylase	-1.70	0.016	Protein modification
19	*RBAM003700*	hypothetical protein	-1.77	0.038	Metabolism of amino acids
20	*accC*	acetyl-CoA carboxylase	-1.83	0.009	Metabolism of lipids
21	*purH*	phosphoribosylaminoimidazolecarboxamide formyltransferase/IMP cyclohydrolase	-2.67	0.005	Metabolism of nucleotides

a) FZB42 + root exudates/ FZB42 – root exudates.

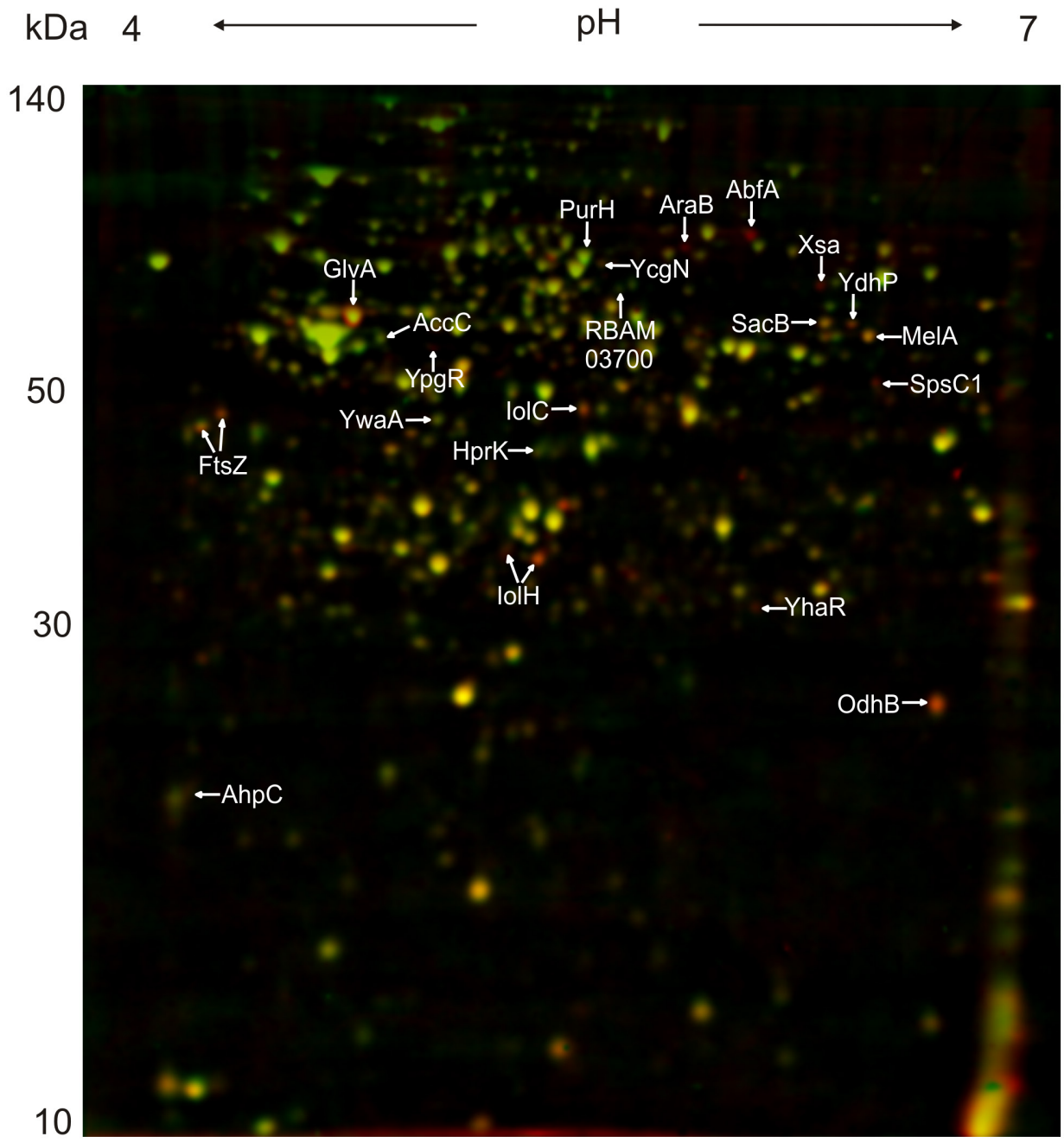

Figure 25 Dual channel image of the representative analytical gels of *B. amyloliquefaciens* FZB42 cells grown in 1C medium. The 2-D protein pattern of cells grown without addition of root exudates (green) was compared to the pattern of cells grown in presence of root exudates (red).

2.9 The role of sigma factors and global regulators in response to root exudates

To better understand the mechanisms of regulation, FZB42 strains bearing knock-out mutations in genes coding for four sigma factors (*sigB, sigX, sigV and sigM*) and two global regulators (*degU* and

abrB) were analyzed. To investigate the response of the genes controlled by these regulators to root exudates, the cytosolic proteomes of FZB42 strains bearing mutations in *sigB, sigX, sigV, sigM, degU* and *abrB* genes were characterized as described in Section 2.6.

2.9.1 Transition state regulators

2.9.1.1 The ΔdegU strain

To obtain more information about the function of the DegU-DegS regulon in mediating bacterial responses to root exudates, expression of cytoplasmic proteins was analyzed.

Of a total of 393 protein spots detected in the FZB42 *degU* mutant strain, 46 were missing, while expression of 54 was found to be significantly altered, compared to the wild type (fold change -1.5 ≤ ≥ 1.5, $p \leq 0.05$). 10 of them were previously reported as DegU-S dependent (Tokunaga *et al.* 1994, Ogura and Tanaka 1996, Ogura *et al*. 2001, Mader *et al.* 2002, Amati *et al*. 2004).

The analysis procedure was exact as in case of secretome analysis of *degU* mutant, described in section 2.6.1.1.

According to Figure 26, IolC and IolH are good examples for positive control of DegU. The same true is for the SacB, however, involvement of DegU in expression of gene encoding this proteins is already known. In all those cases root exudates seems to promote concentration of DegU due to higher expression in their presence. In contrary, YwaA and AccC are more expressed in the mutant strain, which could indicate that DegU acts as a repressor and root exudates seems to negatively regulate the transcription of these genes. Several examples of genes being repressed by DegU have been discovered, including ones involved, like AccC, in metabolism of fatty acids (Ogura *et al*.2001).

Table 11 Proteins mediated by DegU and responding to root exudates.

		Fold change	***p*-value**	**Fold change**	***p*-value**	**Functional classification**
Gene	**Function/similarity**	**WT+/WT-**		**degU+/WT+**		

1 *sacB*	levansucrase	1.57	0.013	-207	0.0008	Metabolism of carbohydrates	DegU
iolC	inositol utilization protein	1.84	0.010	-3.05	0.0001	Metabolism of carbohydrates	-
iolH	inositol utilization protein	1.64	0.004	-2.42	0.0458	Metabolism of carbohydrates	-
2 *ywaA*	branched-chain amino acid aminotransferase	-1.55	0.02	2.21	0.001	Metabolism of amino acids	-
3 *accC*	acetyl-CoA carboxylase	-1.83	0.009	3.49	0.018	Metabolism of lipids	-

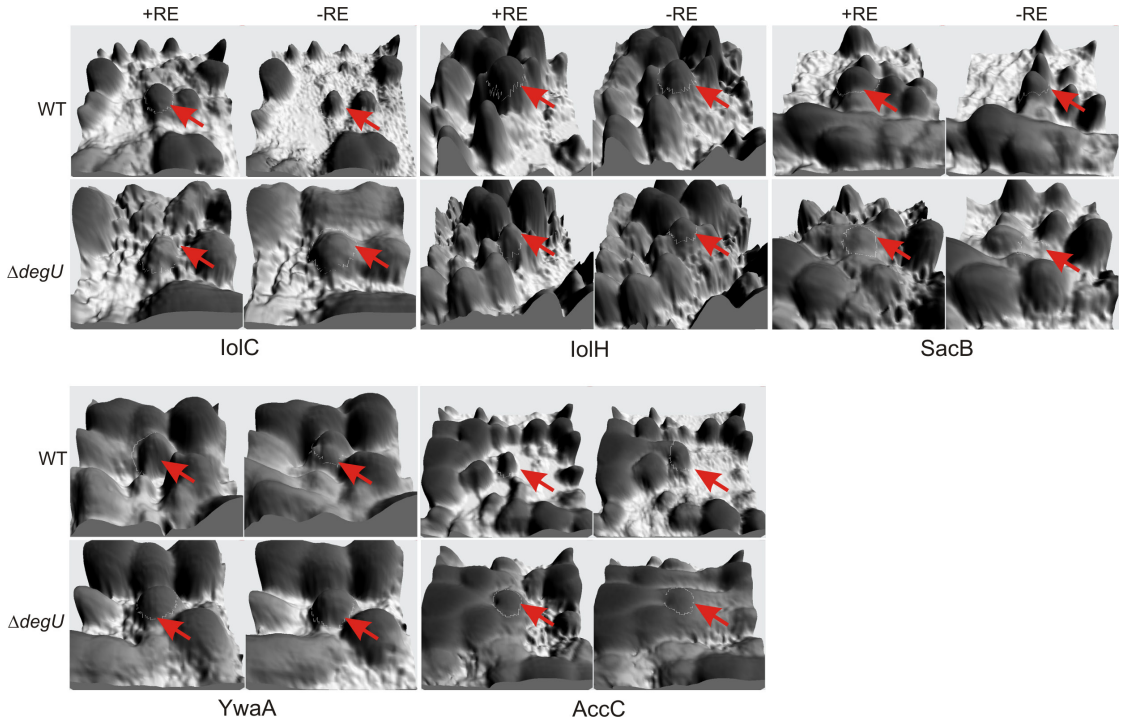

Figure 26 Proteins (indicated by arrows) expressed in a DegU-dependent manner. The images are representative sections of 2-DE gel profiles of proteins extracted from transition growth phase of cells of *B. amyloliquefaciens* FZB42 (WT) strain and the Δ*degU* mutant grown in the presence (+RE) or in the absence (-RE) of root exudates. Gray scale images were obtained from REDFIN software, Ludesi.

2.9.1.2 *The ΔabrB strain*

Disruption of *abrB* gene results in most cases in repression of gene expression. However, AbrB also acts as an activator of some genes (Kim *et al*. 2003, Strauch 1995, Fisher *et al*. 1994). Computer assisted analysis of FZB42 *abrB* mutant proteome resulted in detection of 342 protein spots. Analysis procedures applied were the same as those for DegU mutant. However, applied filter conditions were more stringent, fold change $-2.0 \leq \geq 2.0$, $p \leq 0.05$, to avoid false positive results, since the number of differentially expressed proteins was very high. Expression of 49 proteins was significantly altered when compared to the proteome of the wild type, and 65 proteins were missing, 10 of which were previously reported as a part of the AbrB regulon (Strauch *et al.* 1989). Although the molecular mechanisms of regulation of AbrB in transcription remain elusive, the number of proteins affected by this transition state regulator was the largest among the six transcriptional factors investigated in this study. Interestingly, microarray analysis gave similar results (Fan 2011).

Only three proteins seem to be affected by root exudates via involvement of AbrB. According to Figure 27 PurH and YwaA are inhibited by AbrB, and as it was expected, their expression is lower in the presence of root exudates. Although AbrB acts mainly as a inhibitor, IolC has been found to be AbrB-inducible in presence of root exudates. Several examples of operones and genes activated by AbrB are already known (Hamon *et al*. 2004, Sadaie *et al*. 2008, Strauch, 1995)

According to Table 11 and Table 12 DegU and AbrB seem to have similar influence on IoC and YwaA. It is not particularly surprising since those two transition state regulators are known for regulatory overlap.

Table 12 Proteins mediated by AbrB and responding to root exudates.

			Fold change	*p*-value	Fold change	*p*-value	Functional classification	Transcriptional factor
	Gene	**Function/similarity**	**WT+/WT-**		**abrB+/WT+**			
1	*iolC*	inositol utilization protein	1.84	0.01	-2.02	0.020	Metabolism of carbohydrates	-
2	*purH*	phosphoribosylaminoimidazolecarboxamide formyltransferase/IMP cyclohydrolase	-2.67	0.005	2.38	0.0371	Metabolism of nucleotides	-
3	*ywaA*	branched-chain amino acid aminotransferase	-1.55	0.02	2.85	0.001	Metabolism of amino acids	-

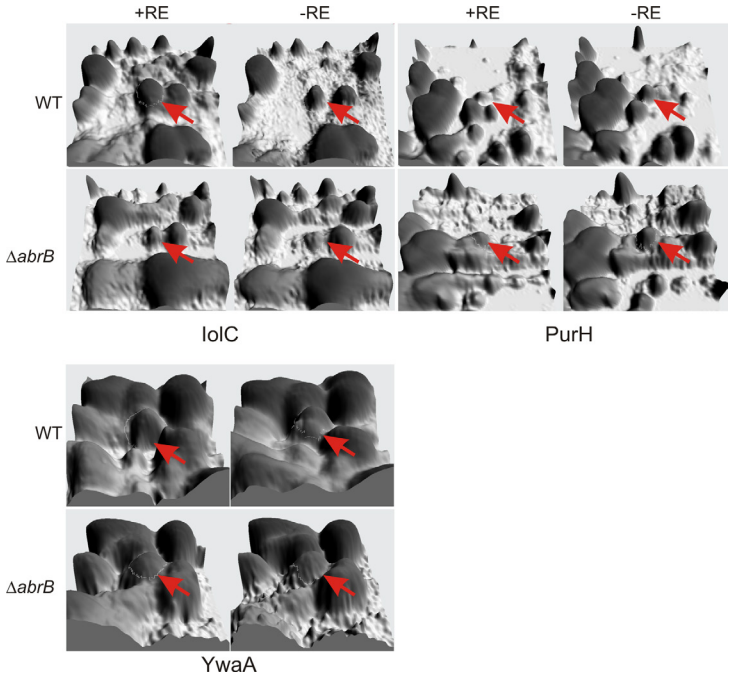

Figure 27 Proteins (indicated by arrows) expressed in a AbrB-dependent manner. The images are representative sections of 2-DE gel profiles of proteins extracted from transition growth phase of cells of *B. amyloliquefaciens* FZB42 (WT) strain and the Δ*abrB* mutant grown in the presence (+RE) or in the absence (-RE) of root exudates. Gray scale images were obtained from REDFIN software, Ludesi.

2.9.2 Heat shock sigma factor *sigB*

In order to gain knowledge about the role of the SigB protein in response of FZB42 to root exudates, the following comparisons were performed. Since sigma factors act as transcriptional activators, only proteins that were down-regulated were considered as being controlled by SigB. Proteins under control of SigB were determined by comparison of Δ*sigB*/ FZB42 WT proteomes, resulting in 26 proteins differentially expressesed (fold change -1.5 ≤, $p \leq 0.05$) among 416 identified protein spots only two proteins were previously reported as SigB dependent: GtaB and YvyD (Petersohn *et al*. 2001, Hoper *et al*. 2005). Subsequently, similarly to the comparisons made in case of the transition state regulators, the data set obtained was compared with the list of FZB42 wild type proteins affected by root exudates (WT+RE/WT-RE) (Table 10).

According to the Figure 28, GlvA and MelA are controlled by SigB. Deletion of a gene coding for SigB completely abolished expression of GlvA and MelA. Additionally, presence of root exudates seems to enhance concentration of SigB due to higher expression of these genes. The GlvA is particularly interesting, since *glvR*, a transcriptional regulator of Glv operon (in *B. subtilis*, Yamamoto *et al*. 2001) has been evidenced to be induced by root exudates and under positive control of SigB in similar microarray approach (Fan 2011).

Table 13 Proteins mediated by SigB and responding to root exudates.

		Fold change	***p*-value**	**Fold change**	***p*-value**	**Functional classification**	**Transcriptional factor**
Gene	**Function/similarity**	**WT+/WT-**		**sigB+/WT+**			
1 *glvA*	maltose-6'-phosphate glucosidase	1.87	0.000	Not expressed		Metabolism of carbohydrates	-
2 *melA*	alpha-D-galactoside galactohydrolase	1.57	0.015	Not expressed		Metabolism of carbohydrates	-

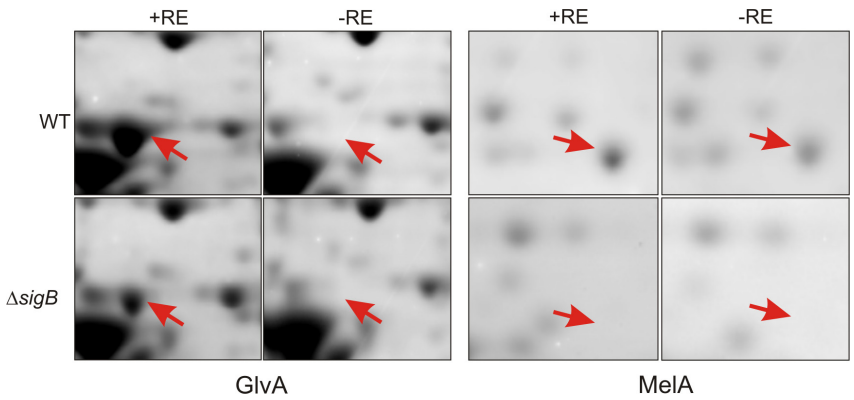

Figure 28 Proteins (indicated by arrows) expressed in a SigB-dependent manner. The images are representative sections of 2-DE gel profiles of proteins extracted from transition growth phase of cells of *B. amyloliquefaciens* FZB42 (WT) strain and the Δ*sigB* mutant grown in the presence (+RE) or in the absence (-RE) of root exudates.

2.9.3 ECF sigma factors SigM, SigV and SigX

The three strains bearing mutation in genes of alternative sigma factors *sigM, sigV* and *sigX* were analyzed in the same manner as *sigB* deletion mutant. There was no involvement in response to root exudates found in the cases of Δ*sigM* and Δ*sigV*. Only one protein was proposed to be altered in response to root exudates via involvement of SigX, the inositol utilization protein C (IolC) (Table 14, Figure 29).

Table 14 Proteins mediated by SigX and responding to root exudates.

		Fold change	***p*-value**	**Fold change**	***p*-value**	**Functional classification**	**Transcriptional factor**
Gene	**Function/similarity**	**WT+/WT-**		**sigX+/WT+**			
1 *iolC*	inositol utilization	1.84	0.01	-2.06	0.000	Metabolism of carbohydrates	-

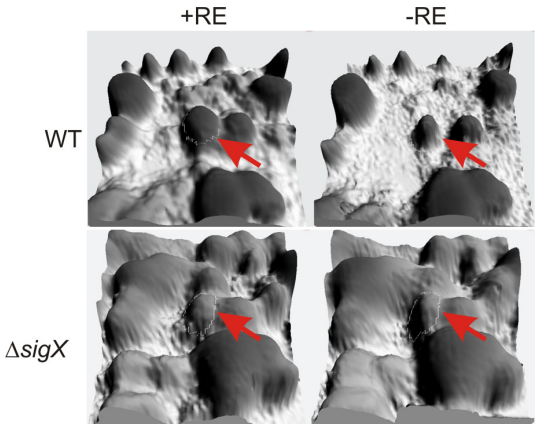

Figure 29 Proteins (indicated by arrows) expressed in a SigX-dependent manner. The images are representative sections of 2-DE gel profiles of proteins extracted from transition growth phase of cells of *B. amyloliquefaciens* FZB42 (WT) strain and the Δ*sigX* mutant grown in the presence (+RE) or in the absence (-RE) of root exudates. Gray scale images were obtained from REDFIN software, Ludesi.

3 Discussion

3.1 Secretory proteins

Secreted proteins play an important role in the adaptation of bacteria to the environment such as, nutrient provision, communication inside the bacterial population, detoxification, and in establishment of either pathogenic (Jungblut *et al*. 1999) or beneficial interactions with other organisms (Meneses *et al*. 2010). Despite the fact that bacilli are known to secrete a huge amount of proteins, there is a dearth of proteomic studies on Gram-positive bacteria. Moreover, only a few studies have investigated the secretion profile of Plant Growth Promoting Rhizobacteria (PGPR) (Buensanteai *et al.* 2008a). At present, 125 proteins have been identified in the extracellular proteome of *Bacillus amyloliquefaciens*. These proteins have various functions, which generally reflect the bacterial lifestyle. In the extracellular proteomes of *Bacillus cereus* (a non-specific pathogen of humans and insects), and *Bacillus thuringiensis* (a pathogen of insects), the majority of secreted proteins are toxins, proteases, or phospholipases (Gohar *et al*. 2005). In contrast, in the secretome of *B. amyloliquefaciens*, around one-quarter consists of proteins involved in the metabolism of carbohydrates. Similar phenomenon was observed in other soil bacterium, such as *B. subtilis* (Tjalsma *et al.*2004).

B. amyloliquefaciens secretes a higher level of proteins in the stationary phase than during the end of transition growth (Figure 4). For instance, expression of most of the degradative extracellular enzymes is repressed during transition growth, but when the cells reach the stationary phase their expression increases. Such mechanisms are dependent on the DegS-U two component system (Msadek *et al.* 1990; Ogura *et al*. 2001), which generally represses the expression of extracellular proteins. When bacteria reach stationary phase (meaning that easily metabolized substrates are already exhausted), degradative extracellular enzymes are expressed, with their main function being to nourish already starving cells.

From 125 identified extracellular proteins, 65 were predicted to be secreted via either Sec or Tat pathways, due to the presence of appropriate N-terminal signal peptide sequences. Unexpectedly, among those 65 proteins, eleven lipoproteins with an aminoterminal cysteine residue were identified. These proteins were found to be involved in transport processes. Interestingly some of the identified proteins were ABC transporters and therefore, their N-terminal domain would be expected to be lipid-anchored in the membrane. It is most likely that these proteins are released from the membrane by "proteolytic shaving" (Antelmann *et al.* 2001; Wolff *et al.* 2007). However, other explanations cannot be excluded, and possibly, prior to the amino-terminal cleavage, those lipoproteins could be actively released from the membrane with the help of the hypothetical Release Factor (RF), as was observed for lipoproteins

of *E. coli* (Yakushi *et al*. 2000). Alternatively, passive leakage of those lipoproteins from the membrane could also have taken place (Antelmann *et al.* 2001).

Several cell-wall related proteins with variable numbers of repetitions with affinity to components of the cell wall additional to the N-terminal signal peptide (Tjalsma *et al.* 2000), were found in the secretome of FZB42. Such proteins were also observed in other bacterial strains such as *B. subtilis* (Antelmann *et al.* 2001; Wolff *et al.* 2007) and *B. licheniformis* (Voigt *et al.* 2006). Interestingly, these proteins were secreted at a higher extent during the transition growth (e. g., CwlO, YodJ, YocH) (Appendix Table 1), indicating more vigorous cell turnover. Alternatively, their presence in the secretome, could be due to the action of extracellular proteases, since in protease-deficient strains cell wall proteins were stabilized (Antelmann *et al*. 2002).

Motifs for translocation via the Tat pathway were found in the signal peptide sequences of three extracellularly identified proteins (containing the canonical twin-arginine motif R-R-XH-H, where H is a hydrophobic amino acid) (Table 1) (Tjalsma *et al.* 2000). In *B. subtilis* those proteins were predicted to posses Tat motifs as well (Antelmann *et al.* 2001). However, only PhoD has been shown to translocate Tat dependently, while the other motifs are apparently not recognized by Tat machinery (Jongbloed *et al*. 2002).

In the extracellular proteome of FZB42, 60 proteins lacking a typical secretion signal were found (Appendix Table 2). Among these proteins were two hook-associated proteins (FliD, FlgK), the hook protein (FlgE) and flagellin (Hag), which are either released from the damaged flagella, or exported via flagellin dedicated machinery for the assembly of flagella (Antelmann *et al.* 2001). It has been previously noted that Hag is not translocated Sec-dependently in *B. subtilis*, because depletion of SecA synthesis did not affect Hag secretion rates (Hirose *et al*. 2000). This finding was further confirmed by Jongbloed, who used sodium azide to inhibit activity of SecA, and found that secretion of FliD and Hag was not disturbed (Jongbloed *et al.* 2002).

The remaining proteins that lack a signal peptide with a clearly cytoplasmic functions, were also found in extracellular proteomes of closely related to FZB42 bacteria such as, *B. subtilis* and *B. licheniformis* (Antelmann *et al.* 2001; Voigt *et al.* 2006), *Paenibacillus larvae* (Antunez *et al*. 2010). In addition, cytoplasmic proteins, such as aldolase, elongation factor G, GroEL, and various dehydrogenases, have also been found in the secretome of other eubacteria such as, group A streptococci (Lei *et al*. 2000), and mycobacteria (Jungblut *et al.* 1999; Rosenkrands *et al*. 2000). It is unclear whether the extracellular presence of those proteins is caused by active secretion or by passive release from the cells during cell wall turnover. It is worthwhile to mention, that more than one-half of identified extracellular proteins of those organisms lacked a typical signal peptide. Since some of the latter proteins of group A streptococci play a role in signaling events between the host and pathogen, it is possible to speculate that some of the equivalent proteins of *B. amyloliquefaciens* may support the establishment of interac-

tions with the plant host and enable survival in the soil and the rhizosphere. Enolase is an interesting example of glycolytic enzyme found to be immunogenic protein and reported as plasminogen activator which binds to the infected host plasminogen activating its proteolytic activity and allowing the bacteria to acquire surface-associated proteolytic activity, constituting an advantage for tissue invasion (Agarwal *et al*. 2008, Sha *et al*. 2009, Antunez *et al*. 2010)

3.2 Involvement of extracellular enzymes in interactions with plant

In this study secretion dynamics of *B. amyloliquefaciens* proteins were investigated, and to our knowledge, this is the first description of a time course secretion analysis of Gram-positive PGPR. The motivation for this study came from the fact that secreted proteins are known to be involved in plant-bacterium interactions (De-la-Pena *et al*. 2008, Zeng and He, 2011). Identification of gene expression patterns at transition and stationary phases, and relation of these changes to important physiological events, is a fundamental issue in the understanding of plant-microbe interactions. Determination of a specific roles for different sets of secreted proteins will help to expand this knowledge. The majority of identified proteins in both growth phases were proteins involved in the metabolism of carbohydrates and amino acids, with a noticeably higher number of extracellular hydrolases secreted in the stationary phase. This might reflect the bacterial adaptation to the shortage of easily metabolized carbon sources in the stationary phase. In addition, microbe-associated hydrolytic enzymes digesting plant cell wall structures, resulting in free oligosaccharides, have been shown to act as elicitors of plant defense (Ebel and Scheel, 1997). In addition to oligosaccharides, the first described elicitors (Darvill and Albersheim, 1984), (poly)peptides, glycoproteins and lipids, were also shown to trigger initiation of plant pathogen defense (Nurnberger *et al*. 2004). Summarizing, the secretion of extracellular enzymes by *B. amyloliquefaciens* probably contributes to biocontrol of pathogens, by the induction of the plant innate defense response or directly by disturbing integrity of their cell envelope.

In addition, a higher number of proteins involved in detoxification was found in the transition phase. The presence of proteins detoxifying oxygen radicals in the extracellular proteome of phytopathogens was connected with the neutralization of the oxidative burst that plants use to challenge infecting bacteria (Soto *et al*. 2006). Thus, a higher level of expression of superoxide dismutase (SodA) and alkyl hydroperoxide reductase (AhpC) at transition phase may indicate that at this point of growth, bacteria are prepared to overcome oxidative stress. SodA is responsible for the inactivation of superoxide anion, and moreover has been shown to be a virulence factor for some phytopathogenic bacteria (Soto *et al.* 2006). In this light, secretion of these proteins by *B. amyloliquefaciens* could be important for es-

tablishment of the interactions with plants and its maintenance in the rhizosphere (Meneses, *et al.* 2010).

In the secretome of *B. amyloliquefaciens* several proteins are generally assumed as Pathogen Associated Molecular Patterns (PAMPs), to which plants have developed perception systems. These include flagellin, elongation factor Tu (TufA), and cold shock proteins (CspBCD). Flagellin has been shown to trigger plant-defense-associated reactions in plants as diverse as *Arabidopsis* and tomato (Felix *et al*. 1999). This phenomenon was first observed in virulent bacteria, whose flagellin induces defense responses much faster and at higher level (Lindgren 1997). Elongation factor Tu (TufA), which is essential for protein translation, has been reported to be a potent elicitor of defense responses and diseases resistance in *Arabidopsis* (Kunze *et al*. 2004).

Expression of Cold Shock Proteins (CSPs) at transition phase was approximately six times higher than at the stationary phase (Appendix Table 2). It was suggested that CSPs are a novel class of bacterial elicitors, for which *Solanales* have developed specific and sensitive chemoperception systems (Felix and Boller 2003). Although CSPs lack signals for secretion, accidental cell lysis during culturing would probably not generate so high amounts of these proteins, and an alternative mechanism of protein release can be taken under consideration. Summarizing, at the transition state of growth when bacteria decide whether to establish the symbiotic relationship with plant, elicitors are forms of signaling molecules probably involved in initial recognition of bacteria by plants (Gomez-Gomez and Boller 2000).

Plants respond to PAMP molecules by induction of ion-flux across the plasma membrane, increased intracellular Ca^{2+} concentration, oxidative burst, mitogen-activated protein kinase activation and major transcriptional changes. Similar responses have been observed in epidermal cells of legume roots as a result of interaction with Gram-negative symbiotic bacteria. It is tempting to speculate that the extracellular presence of these proteins contributes to the overall beneficial effects that *B. amyloliquefaciens* FZB42 exerts on plants; in this particular case, induction of innate plant immunity.

Presented results also show secretion of proteins without assigned functions mainly, but not exclusively, in the stationary phase. Further studies need to be carried out to clarify their functions, since the extracellular proteins are in direct contact with environment and through their involvement, the interactions with host plants are established.

3.3 Cytosolic proteins

A cytosolic protein reference maps of *B. amyloliquefaciens* cells grown in 1C and minimal media were created. It is particularly important to prepare such maps, because they facilitate the study of differential expression of proteins under conditions of interest. Transition state of growth was chosen as the most suitable time for cell collection, because this growth phase seems to be a crossroad in the life cycle of the cell. In this particular phase the cell decides which metabolic state to enter. In laboratory conditions the transition state is quite transitory, but in the natural soil habitat of FZB42 (where nutrients are not always available), the growing state of the bacterial cells are probably more akin to the transition state than to rapid logarithmic growth in rich media (Phillips and Strauch 2002).

The formation of the reference map began with the creation of the theoretical proteome based on the genomic sequence (Figure 16). According to the predicted proteome, soluble proteins exhibited theoretical molecular masses ranging from 7 kDa to 560 kDa. Acidic proteins with an isoelectric point (p*I*) between 3.2 and 7 constituted almost 58% of the theoretical proteome, while 27% of proteins displayed p*I* greater than 9, and only 14% of proteins were distributed across the 7 to 9 p*I* range. This bimodal repartition of proteins along the pH gradient is a general property of the bacterial protein complement (Schwartz *et al.* 2001). Since almost two thirds of the artificial proteome is constituted by acidic proteins, and the majority of them possessing molecular masses between 10 kDa and 130 kDa; a pH gradient between 4 - 7 together with a 12.5% polyacrylamide gel were chosen as the standard analytical window, resulting in identification of 461 different proteins obtained from the cytosol of cells grown in 1C medium.

According to the peptide mass fingerprinting (PMF) data, each protein was identified 1.53 times, indicating that posttranslational modification (PTM) occurs in *B. amyloliquefaciens*. This is in agreement with published values for other prokaryotes (Maillet *et al*. 2007; Chao *et al*. 2010), and is within the lower range reported for bacteria. It is worth noting, that almost 60% of multiple spots detected on the analytical gel were horizontal isoforms. The most prominent examples are class III stress-response-related ATPase (CplC), transketolase (Tkt) and pyruvate carboxylase (PycA). These proteins were also detected as multiple horizontal spots in other organisms and proven to be posttranslationally modified by phosphorylation (Buttner *et al.* 2001; Bendt *et al*. 2003). Another reason for multiple spot occurrence could be artificial chemical preparations (Buttner *et al.* 2001). A group of six proteins with apparently altered molecular weights were found, which could be a result of fragmentation caused by proteolitic cleavage or oligomerization (Table 15)

Table 15 Proteins associated with multiple spots.

	Protein	Number of isoforms	Modification[a]
1	AcsA	2	C
2	AhpC	2	C
3	AlaS	2	C
4	CitB	3	C
5	CitZ	2	C-M
6	ClpC	2	C
7	DhaS	2	C-M
8	DnaK	2	C
9	FabI	2	M
10	FtsZ	2	C
11	GidA	2	C-M
12	GlmS	2	C
13	GlnA	2	C
14	GlyA	3	C-M
15	GuaA	2	M
16	IolH	2	C
17	Kbl	2	C
18	Mdh	3	C
19	OdhB	3	C-M
20	PurA	3	C-M
21	PurF	2	C-M
22	PurH	2	C-M
23	PycA	3	C
24	RocA	5	C-M
25	RocD	2	M
26	RocF	2	C
27	SdhA	3	C
28	SecA	3	C
29	SodA	2	C
30	SucA	3	C
31	SucA	3	C
32	SucC	3	C
33	Tig	2	C
34	Tkt	2	C
35	TpiA	2	M
36	YcgN	2	C
37	YdcI	2	M
38	YhfE	3	M
39	YjbG	2	C
40	YkgB	2	C-M
41	YlaG	2	C
42	YlyB	2	C-M
43	YqiL	2	C

a) The character of proteins' modification: C, charge only; M, molecular weight only; C-M, charge and molecular weight.

The reliability of identified proteins was further validated by comparison of theoretically predicted p*I*s and MWs with experimentally observed values. Figure 30 shows a scatter diagram, where theoretical

p*I* and MW values were plotted against those observed on a 2-DE gel. Overall, reasonable correlation between predicted and observed p*I*s (Figure 30A) and MWs (Figure 30B) were found, particularly for the MW values (r^2=0.91), suggesting, that relatively little non-specific proteolitic cleavage during protein preparation and later separation with 2-D electrophoresis occurred. All together, 45 proteins were outside the window of ± 10 kDa and p*I* range of ±0.5. A closer analysis of these outliers, revealed large discrepancies between observed and theoretical values for proteins involved in synthesis of polyketides as macrolactin (MlnA) (MW and p*I* values) and difficidin (DfnY) (MW value), and bacilysin (BacD) (MW value), indicating strong modifications after translation. An interesting example of large discrepancies between predicted and observed p*I* are eight ribosomal proteins, the β subunit of RNA polymerase (RpoC), an oligopeptide ABC transporter (OppA), a thiamin biosynthesis homolog (YkpB), and hypothetical protein (YabE). While the calculated p*I* of those proteins ranged from 8.7 to 10.3, the observed p*I* is much lower, between 4.35 and 6.81. The correlation co-efficient improves from 0.458 to 0.816 by removing these data points from the overall calculation. At the same time, there is no correlation between these 12 data points when their predicted p*I*s are plotted against the observed p*I* values.

An interesting example of PTM affecting the molecular weight, is fragmentation of the RNA polymerase, β-subunit (RpoC), with a detected MW value almost two times lower than the predicted one. This protein was most probably subjected to proteolitic cleavage, which influences the size of the protein and does not dramatically affect the overall protein net charge, which has been previously observed in other bacteria (Langen *et al*. 2000; Lock *et al*. 2001).

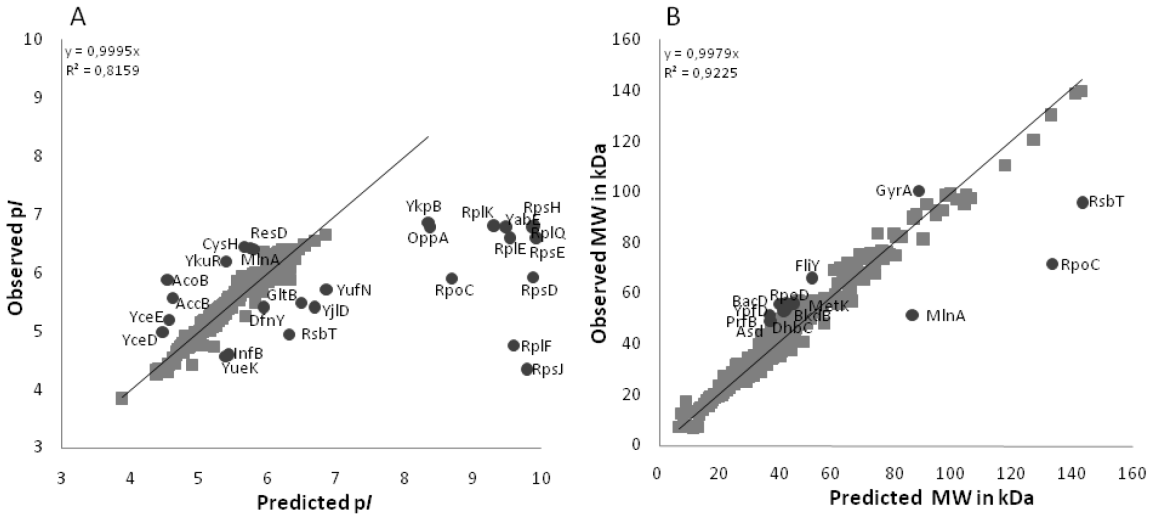

Figure 30 Comparison of experimentally determined and predicted p*I* (A) and MW (B) values of identified protein spots from *B. amyloliquefaciens* FZB42. For the calculation of correlation co-efficient of the p*I* values the 12 outliers were excluded.

The CAI values of all ORFs of *B. amyloliquefaciens* were calculated (Figure 19) and further confirmed by cellular abundance of identified proteins (Table 16). Proteins encoded by genes with a CAI value above 0.5 account for 83% of the predicted proteome, and 96% of the 461 identified proteins. The CAI value of approximately 90% of the 50 most abundant proteins was above 0.6. Evaluation of highly abundant proteins revealed that most posses housekeeping functions mainly in glycolysis, tricarboxylic acid cycle (TCA), amino acid metabolism or as a component of translation apparatus in the cell. A comparison of 50 of the most abundant proteins of *B. amyloliquefaciens* to the most abundant proteins from other bacteria has been performed. The highest similarity to *B. subtilis* was observed (Buttner *et al*. 2001; Eymann *et al*. 2004). A high abundance of proteins degrading plant originated carbohydrates (GlvA - maltose-6'-phosphate glucosidase, IolH - involved in metabolism of inositol), may suggest the importance of a nutritional strategy that is based on an external supply of plant derived compounds. The function for only one of the 50 most abundant proteins is unknown (YxjG), indicating that the most important enzymes for a living cell have been discovered.

Table 16 Summary of the most abundant cellular proteins in *B. amyloliquefaciens.*

Protein	**% Amount**[a]	**CAI**	**Functional class**
TufA	3.75	0.86	Translational apparatus
Mdh	1.68	0.69	TCA cycle
Eno	1.58	0.82	Glycolisis
RocA	1.45	0.57	Amino acid metabolism
RplL	1.43	0.85	Translational apparatus
FusA	1.42	0.79	Translational apparatus
GlvA	1.34	0.57	Specific pathway
CitB	1.34	0.67	TCA cycle
OdhB	1.26	0.61	TCA cycle
FbaA	1.10	0.78	Glycolisis
SucA	1.08	0.57	TCA cycle
Icd	1.06	0.69	TCA cycle
GroEL	1.05	0.76	Protein quality control
Tig	1.04	0.79	Protein quality control
Tsf	0.98	0.83	Translational apparatus
Hag	0.87	0.79	Other
SodA	0.87	0.76	Other
AtpA	0.85	0.69	Other
PdhD	0.83	0.73	Glycolisis
PurH	0.75	0.59	Nucleotides metabolism
DhaS	0.74	0.56	Coenzymes metabolism
RplJ	0.73	0.81	Translational apparatus
RocF	0.72	0.54	Amino acid metabolism
Tkt	0.72	0.67	Other
GapA	0.70	0.86	Glycolisis

Bcd	0.68	0.61	Lipid metabolism
CspD	0.67	0.85	Other
GlyA	0.67	0.68	Amino acid metabolism
RocD	0.65	0.61	Amino acid metabolism
YcgN	0.65	0.67	Amino acid metabolism
Pgi	0.64	0.69	Glycolisis
YqiI	0.64	0.57	Cell wall
SucD	0.62	0.65	TCA cycle
FabI	0.62	0.68	Lipid metabolism
TpiA	0.62	0.73	Glycolisis
GlnA	0.60	0.74	Amino acid metabolism
DnaK	0.58	0.75	Protein quality control
PdhB	0.57	0.74	Glycolisis
YurU	0.57	0.72	Sulfur metabolism
PdxS	0.55	0.73	Coenzymes metabolism
AtpD	0.55	0.68	Other
IolH	0.54	0.63	Specific pathway
PnpA	0.53	0.66	Nucleotides metabolism
SdhA	0.52	0.60	TCA cycle
AhpC	0.52	0.87	Other
YdjL	0.49	0.69	Specific pathway
Ndk	0.47	0.62	Nucleotides metabolism
YxjG	0.47	0.60	Function unknown
IlvC	0.46	0.68	Amino acid metabolism
PdhC	0.46	0.75	Glycolisis

a) The percentage of protein abundance corresponds to the percentage of specific spot volume related to the total spot volume in the cellular proteome. Quantification of spots volume was performed with Ludesi software.

The majority of the most abundant proteins posses CAI values above 0.5. In addition, direction of the transcription of those genes, in most cases, corresponds to the direction of the FZB42 replication (Figure 31)

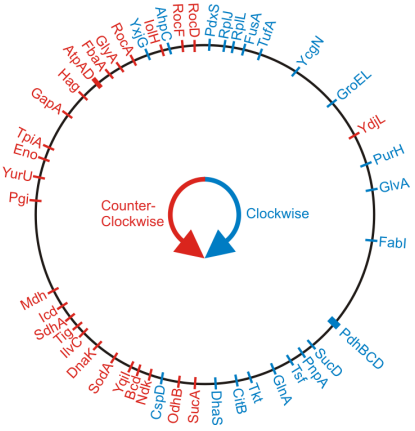

Figure 31 The graphics represents chromosomal localization of the genes encoding for the 50 most abundant proteins of *B. amyloliquefaciens* during transition growth. Gene names are placed on the circle that is representing the chromosome. The directions of transcription and replication are blue and red.

3.4 Proteome profiling of *B. amyloliquefaciens* in response to maize root exudates

Most of the proteomics investigations that have been conducted on soil bacteria were aimed at modeling the symbiotic relationship between the nitrogen fixing rhizobia and legume plants. The proteomic approach was also extensively used to decipher the bacterial traits active during the nodule formation on other dicot plants like *Alnus* induced by *Frankia alni* (Alloisio *et al*. 2007), or proteomic prospective of *Sinorhizobium meliloti* metabolism in the root nodule of *Medicago truncatula* or *Melilotus alba* (Djordjevic 2004). Microorganisms establishing pathogenic interactions with plants has recently gained attention, with large bodies of literature deciphering this type of interaction emerging (Andrade *et al*. 2008; Lauzier *et al*. 2008). To my knowledge, only one proteomic study concerning interactions between root exudates of canola and symbiotic plant associated Gram-negative bacterium *P. putida* strain UW4 has been performed (Cheng *et al*. 2009).

To unveil the basis of the communication pathways between Gram-positive PGPR and plants, the changes in organization of proteins expression in the cytosol and secretome of *B. amyloliquefaciens* in response to the compounds exuded by maize roots was evaluated. Proteins were isolated either from cells or growth medium (extracellular proteins) at transition phase and 2-D gels were prepared. Additionally, extracellular proteome of cells grown at stationary phase was prepared. Root exudates influenced similar proportions of extracellular proteins, 13% in the secretome of cells harvested at transition phase, and 17% of proteins from cells harvested at stationary phase. Lower percentage of proteins changed their expression in response to root exudates in the intracellular proteome of *B. amyloliquefaciens* (4.5% significantly altered). Similar proportions of up- and down- regulated cyto-

solic proteins of *P. putida* UW4 was observed in response to root exudates of *B. napus* (Cheng *et al*. 2009), although the total number of altered proteins was higher and reached approximately 20% of identified proteins. On the other hand, transcriptomics analysis of FZB42 revealed 8.2% of genes affected by supplementation of root exudates, and similarly to the presented work, a higher proportion of them were up-regulated (Fan 2011).

3.4.1 Proteins involved in nutrient utilization

It is thought that a microorganism's capacity to utilize carbohydrates presumably reflects the availability of such substrates in its habitat (Chhabra *et al*. 2003). Therefore, it is not surprising that bacterial proteins involved in utilization of nutrients and their transport were induced by root exudates, since they represent an additional source of carbon. During exponential growth (when the preferred carbon source is easily accessible), genes coding for utilization of secondary metabolites are subjected to Carbon Catabolite Repression (CCR) mechanisms, which manipulate bacterial metabolism to utilize easily metabolized carbon sources, guaranteeing fast growth and competitive success with other microorganisms (Gorke and Stulke 2008).

Prior to utilization of complex carbohydrates as a carbon and energy source, glycoside hydrolases must digest the carbon backbone, so the side chain glycosidic linkages are hydrolyzed to the extent needed for binding, transport and intracellular utilization. The proteomics results presented revealed that in the presence of plant root exudates, expression of hydrolytic glycosidases is induced. One of such glycoside hydrolases is extracellular arabinase (AbnA), which catalyzes the endohydrolysis of 1.5-alpha-arabinofuranosidic linkages in 1.5-arabinans. In addition, proteins depolymerizing the extracellular glucomamman, beta-1.4-mannanase (YdhT) and xylan, endo-xylanase (YnfF), (St John *et al*. 2006), have been induced by addition of root exudates. These results obtained from transition growth indicate that CCR has been abolished since those proteins are subjected to its mechanisms, and YdhT and AbnA are substrate specific inducible (Raposo *et al*. 2004; Sadaie *et al*. 2008), while YnfF is negatively regulated by AbrB (the transition state regulator) (Chumsakul *et al*. 2010).

As a result of digestion, smaller units of polysaccharides, in a suitable size for binding can be transported into the cell. Unfortunately, not many cell wall located permeases of the ABC-type transporters were identified, probably due to their strong hydrophobic nature (Antelmann *et al*. 2002). Proteomics of the cell wall proteins should be performed to gain more knowledge about intracellular transport of such macromolecules. However, several ABC-type transporters were affected by the root exudates, especially in the stationary phase. Surprisingly, proteins involved in transport of iron were down-regulated. It would be expected that these particular proteins would be induced in this growth phase since iron is a growth limiting ion required as a cofactor for many enzymes involved in the mainte-

nance of basic metabolic pathways. On the other hand, too high concentrations of iron in the cell can result in production of oxygen radicals that can cause severe damages to the majority of cellular biomolecules (Touati 2000). Exponentially growing bacteria require iron for rapid multiplication, and expression of FZB42 genes involved in its acquisition have been induced by root exudates in this growth phase, which was also revealed by a transcriptomic approach (Carvalhais 2010). Subsequently, as the cells reach stationary phase (and do not actively propagate), the intracellular free iron content becomes high and, therefore, the proteins involved in transportation of extracellular iron have to be reduced to prevent toxicity (Merrell *et al*. 2003).

The ABC-type phosphate transporter, PstS, was strongly up-regulated in the cells grown at stationary phase. Similarly, the proteomics of *B. subtilis* showed induction of this protein during phosphate starvation (Antelmann *et al*. 2000). This indicates that at stationary phase phosphate is a limiting nutrient. At the same time, active secretion of phytase begins at stationary phase (Figure 5,Table 2), what is not surprising since *phyC* gene of cognate *B. amyloliquefaciens* FZB45 is controlled by the PhoPR two-component system induced under phosphate starvation (Makarewicz *et al.* 2006). This suggests that phytase, like other extracellular proteases and depolymerases, acts as a 'scavenger' enzyme after exhaustion of rapidly metabolized nutrient sources (Idriss *et al*.2002). Therefore, it can be concluded that phytase secretion affects both bacteria and plants, by mobilization of the phosphorus fixed as insoluble organic phytate, a principal storage form of phosphorous in plant tissues, especially seeds (Richardson *et al*. 2001). Incorporated mobilized phosphorous fulfills the nutritional demands of both bacteria and the plant, which contributes to *B. amyloliquefaciens* FZB42 plant growth promoting activity.

In general, secretion of proteins transporting amino acids was more intense at stationary growth phase than at transition. In addition, supplementation of root exudates induced expression of oligopeptide binding protein OppA (transition phase) and AppA (stationary phase), which is consistent with similar transcriptomic analysis of FZB42 (Fan 2011). OppA is a component of the oligopeptide permease, and is involved in the uptake of small oligopeptides (regardless of amino acid sequence specifity) to utilize as a carbon and nitrogen source (Tame *et al*. 1994). Interestingly, the Opp transport system is also required for the import of cell-to-cell small signaling compounds (Lazazzera 2001).

Surprisingly, secretion of an enzyme that depolymerizes chitin into N-acetylglucosamine and chitobiose was repressed by root exudates at transition growth. Chitin is the second most ubiquitous polymer in the soil after hemicellulose, and serves as a carbon and nitrogen source for some bacteria (Fritsche *et al*. 2008; Juarez-Jimenez *et al*. 2008; Rhodes *et al*. 2009), however, the hydrolysis process is labor-intensive and not very cost effective due to the low yield (Murao 1992). Interestingly, chitosan (a product of chitin hydrolysis), elicits systemic plant resistance against fungi when applied as a seed treatment or soil amendment (Benhamou 1994). Consequently, it is tempting to speculate that in general the secretion of chitosanase by FZB42 exerts beneficial effects on plants by fungi suppression and

induction of plant systemic resistance. Lower secretion rates may be due to the presence of various sugars, especially glucose added with root exudates, as observed in the case of *Collimonas* species (De Boer *et al*. 1998). Prolonged incubation of FZB42 leads to exhaustion of easily metabolized sugars, thus, secretion of chitosanase is not repressed by root exudates at the stationary phase. However, a putative chitin binding protein RBAM 1754 is strongly induced by root exudates in this growth phase indicating that depolymerization of chitin has occurred.

Starch is the most common reserve carbohydrate in seeds, and is mobilized to provide energy for seed germination and early seedling growth (Smith *et al*. 2003). During seed imbibition, starch is converted in-to forms that are readily transportable to sites where they are required, and part of this leaks to the surrounding environment. Depolymerization of starch results in maltose (Schonert *et al*. 2006), and its concentration in seed exudates was approximately 700 times higher than in root exudates (Carvalhais 2010). In the present study, collected substances exuded by plants were not separated in to seed and root exudates, instead were pooled together, what reflects the natural state of exuded compounds accumulated in the rhizosphere. Consequently, high amounts of maltose are very likely to be present in the exudates applied in this study. Both, proteomics (Table 10) and transcriptomics (Fan 2011) approaches have shown strong induction in expression of proteins/genes involved in metabolism of maltose. During transition growth, expression of maltose-6'-phosphate glucosidase (GlvA), which converts maltose to glucose and glucose 6-phosphate in the cytoplasm, was almost two times higher when the plant exudates were added in-to the growth medium. It is tempting to speculate that active secretion of α-amylase during transition and stationary phases, coupled with the ability to convert maltose to glycolysis intermediates, favors *B. amyloliquefaciens* FZB42 in the rhizosphere, allowing it to exert beneficial effects on plants by different modes of actions (Chen *et al*.2007).

The roots of 13-15 day old maize plants excrete sugars; with sucrose, fructose and glucose being the main components (Kraffczyk 1984; Carvalhais 2010). Most probably for that reason, sucrose inducible cytoplasmic levansucrase (2.6-β-D-fructan-6-β-D-fructosyl-transferase) (Marvasi *et al*. 2010) encoded by *sacB*, was found up-regulated by root exudates at transition growth phase (Table 10). *B. subtilis* which shares 96% of amino acid identity with *B. amyloliquefaciens* levansucrase, is able to catalyze both sucrose hydrolysis and levan polymerization, from glucose and fructose residues (Meng and Futterer 2003). The secretion of levansucrase via the Sec pathway to the environment is probably followed by accumulation of this enzyme in the cytoplasm, where the hydrolysis of sucrose provides the major intermediaries for glycolysis. It has been suggested that in *Pseudomonas* spp., levan forms a capsule that protect against bacteriophages, attracts solutes, creates an osmotic gradient, and helps to prevent cell desiccation (Paton 1960). More importantly, however, is that this polysaccharide when synthesized by *Paenibacillus polymyxa* CF43 was reported to facilitate the aggregation of root-adhering soil on wheat plants, thus improving soil structure (Bezzate *et al*. 2000). Improved soil aggregation in the root surroundings is important for nutrient uptake by plants. The closer to the surface

of the roots, the more soil structure can be modified. This can occur either directly by rhizodeposition, or indirectly by stimulation of microbial activity in the rhizosphere (Bezzate *et al*. 2000). Polymerization of levan by *B. amyloliquefaciens* could be another beneficial effect that this bacterium exerts on plant nutrition. Further experiments need to be performed to test this hypothesis.

In the presence of maize root exudates, bacterial cells grown in transition phase showed induction of cytoplasmic proteins involved in the catabolism of *myo*-inositol. Various functions have been attributed to *myo*-inositol, including signaling roles in animal and plant cells (Loewus 2000). Additionally, several bacterial species were reported to grow efficiently with inositol as a main carbon source, including *Sinorhizobium meliloti* (Galbraith *et al*. 1998), *Lactobacillus casei* BL23 (Yebra *et al*. 2007), and *B. subtilis* (Yoshida *et al*. 2008). Interestingly, functional catabolic pathways of inositol were recently reported to be essential for the Gram-negative nodulating bacterium *S. meliloti* during the competition in colonization of alfalfa roots (Kohler *et al*. 2010). This finding extends the role of inositol beyond that of a nutrient source, and sheds a new light on its signaling role in the symbiotic relationship. Results presented in this study, and confirmed by similar transcriptomic approaches (Carvalhais 2010; Fan 2011), suggest that it is possible that inositol has a signaling function in the establishment of interactions between plants and *B. amyloliquefaciens*.

Surprisingly, the proteomics analysis of proteins secreted by FZB42 at transition growth presented in this study revealed that one of the most strongly induced proteins in response to root exudates was acetolactate synthase (AlsS) (Table 4). This protein, under oxygen-limiting conditions, catalyzes conversion of two pyruvates to acetoin and 2.3-butanediol (Ryu *et al*. 2003). However, AlsS lacks a signal peptide and catalyzes clearly cytoplasmic reaction. There are two possible explanations for its extracellular presence, 1) random cell lysis during culturing, or 2) AlsS playing a significant role in the establishment of interactions with the plant host. Two bacterial strains *Bacillus subtilis* GB03 and *Bacillus amyloliquefaciens* IN937a, that produce a blend of volatile compounds with 2.3-butanediol and acetoin as the main constituents were proved to induce plant ISR (Ryu *et al*. 2004). In light of this fact, it would be proposed that *B. amyloliquefaciens* FZB42 responds to signaling compounds from root exudates by activation of an alternative reductive pathway originating from pyruvate, which results in the production of acetoin and 2.3-butanediol. It is important to mention that a volatile profile emitted by bacteria is diverse and could serve as a taxonomic marker in a microbial system (Scholler *et al*. 2002; Farag *et al*. 2006). However, mechanisms of volatile organic compound emissions by *B. amyloliquefaciens* need to be further examined to confirm this hypothesis.

3.4.2 Proteins involved in amino acid utilization

Although amino acids have been suggested to represent a minor fraction of maize root exudates (Vancura 1964; Kraffczyk *et al.* 1984), a wide range of amino acids can be found, including proline as a major component (Bacilio-Jimenez *et al*. 2002). Accumulation of proline in the elongating primary roots of maize has been linked with plant growth under stress conditions (Voetberg and Sharp 1991; Verslues and Sharp 1999; Spollen *et al*. 2008), and it is possible that the presence of this compound in root exudates is due to active exudation or passive leakage through the root cap cells. Several studies reported induction of proline catabolizing genes in Gram-negative rhizobacteria as a result of interactions with plants (Jimenez-Zurdo *et al*. 1997; Vilchez *et al*. 2000), but for the first time, a similar response has been observed in Gram-positive bacteria. A protein involved in the catabolism of proline to glutamate, 1-pyrroline-5-carboxylate dehydrogenase (YcgN), was induced in the intracellular proteome of *B. amyloliquefaciens* cells grown at transition phase by the presence of root exudates (Table 10). A putative proline-specific permease gene of FZB42 showed similar responses to root exudates in the transcriptomic approach (Carvalhais 2010). Surprisingly, analysis of 2-D gels of proteins secreted by cells at the same growth phase, showed significant reduction of YcgN expression (Table 4). However, YcgN is not considered a secretory protein due to its cytoplasmic function, and does not possess either classical or nonclassical signal peptide sequence, which would direct it to be transported across the bacterial cell wall (Appendix 2). The repressing effect of root exudates is probably due to the fact that some of the YcgN copies are needed intracellularly for conversion of proline to glutamate, resulting in their lesser extracellular presence. However, it was hypothesized that essential genes involved in basic metabolic functions, originally responsible for carbon and nitrogen acquisition, have evolved into genes encoding for functions relating to specific interactions with plant roots (Peters and Verma 1990).

3.4.3 Proteins involved in chemotaxis and motility

During transition growth phase, proteins related to chemotaxis and motility: flagellar hook-associated protein II (FliD) and flagellin protein (Hag) (extracellular proteome Table 4) were induced in the presence of root exudates. Flagellin proteins are recognized by most of the plasma-membrane-localized pattern recognition receptors, and are thought to elicit a plant basal defense against potential pathogens (Zeidler *et al*. 2004), which may contribute to *B. amyloliquefaciens* biocontrol competence. Higher expression of flagellin related proteins in the presence of root exudates could perhaps be due to plant derived signaling molecules, than due to nutritional possibilities given by supplemented root exudates. Bacterial cultures enriched with root exudates are subjected longer to the CCR, because the main compounds of maize root exudates are easily metabolizable carbon sources such as fructose, glucose,

maltose and sucrose (Carvalhais 2010). The ability of FZB42 to recognize plant-emitted signals is a crucial step in establishment of plant-microbe cross-talk (Bais *et al*.2006), and this ability would probably contribute to its competence in the rhizosphere.

3.4.4 Other proteins

In general, repression of extracellular enzymes involved in the sugar phosphotransferase system either at transition (PtsI) (Table 4) or stationary (PtsH) (Table 5) growth was observed. This indicates that particular substrates are absent in the extracellular environment. However, it remains questionable, why those enzymes without signal for secretion in their N-terminal sequence are found in the extracellular surroundings of bacteria.

3.5 Response to root exudates via possible involvement of global regulators and sigma factors

In the present study, six genes coding for proteins involved in regulation of transcription were tested using a proteomics approach. The aim of this work was to determine if the response of FZB42 to root exudates is mediated via the tested transcriptional regulators.

Proteomics is a powerful high through-put method for detecting changes in protein organization caused by various factors, such as deletion of a regulatory gene. At the same time, this method is prone to both false positive or false negative results. Consequently, involvement of regulatory proteins in response to root exudates had to be interpreted with caution. Presented results reveal that σ^V does not participate in gene transcription regulation during establishment of plant-microbe interactions. This finding was confirmed by similar microarray approach, where only four proteins were proposed as responding to root exudates via σ^V involvement. In general, ECF sigma factors appear not to be strongly involved in these processes. Only one protein beta-1.4-mannanase (YdhT) could be proposed as regulated by σ^M and σ^X (Table 8, Table 9), although this needs to be further experimentally confirmed. Such regulatory overlap and functional redundancy of ECF sigma factors was also observed in *B. subtilis* (Mascher *et al*. 2007), and is probably due to evolutional adaptability of bacteria to constantly changing external conditions.

Although deletion of *sigB* coding for heat shock sigma factor did not affect many genes in both, proteomic and microarray approaches, the existing overlap in results sheds a new light on genes regulation. Protein coded by a gene belonging to the glv operon involved in maltose uptake and utilization has been shown to be regulated by σ^B in present work. Additionally, transcriptomic approach revealed

similar results for *glvR* (Fan, 2011), a transcriptional activator of this operon. Thus, in can be concluded that glv operon of *B. amyloliquefaciens* is under control of σ^B, and according to the Figure 28 and transcriptomic approach (Fan, 2011), root exudates seems to enhance concentration of SigB.

Global regulators appear to be more involved in responses to root exudates. Regulation via involvement of root exudates has been evidenced for: Csn, SacB and OppA (DegU), YnfF (AbrB), what is in agreement with previous findings (Mader *et al*. 2002; Chumsakul *et al*. 2010; Borgmeier *et al*. 2011). Extracellular protein of unknown function RBAM 1764 is proposed to be negatively controlled by DegU via involvement of root exudates. However this hypothesis needs to be further confirmed.

4 Materials and Methods

4.1 Bacterial strain and culture conditions

A single overnight colony of *Bacillus amyloliquefaciens* FZB42 strain was cultivated at 24°C, under 210 r.p.m. in liquid 1C medium containing 0.7% tryptone, 0.3% soy peptone (papain digest), 0.1% glucose and 0.5% NaCl or in minimal medium containing 70 mM K_2HPO_4, 30 mM KH_2PO_4, 25 mM $(NH_4)_2SO_4$, 0.5 mM $MgSO_4$, 10 µM $MnSO_4$, 22 mg/L ferric ammonium citrate, 6 g/L sodium succinate, 8 g/L potassium glutamate (Stulke 1993). Bacterial growth was monitored by measurement of the OD at 600 nm. When the optical density achieved 1.0, an aliquot corresponding to the 1% of final volume was added in to the main culture 1C medium, supplemented with 10% of soil extract and, if necessary, root exudates added into the culture up to a final concentration of 250 µg dry weight per mL. Soil extract was prepared as follow: 500g of soil was mixed with one liter of distilled water, incubated for 45 min at 98°C and filtered. The sterility was controlled by depositing 200 µL of soil extract on LB agar plate and incubated at 37 °C for 24 h.

4.2 Sterilization of maize seeds and collection of root exudates

Maize seeds (*Zea mays* L. var. Agadir) were shaken for 3 min in 96% ethanol, 30 min in 5% sodium hypochlorite solution and rinsed twice with sterile distilled water (SDW). Such prepared seeds were left to soak in SDW for 4 h at room temperature. Subsequently, maize seeds immersed in SDW were placed for 10 min at 60°C water bath, what was followed by twice rinse with SDW. After surface sterilization, 10 seeds were placed, embryo upside, on Petri dish covered with Whatman paper soaked with SDW. Seeds were kept in dark at room temperature, the moisture of Whatman paper was checked every 12 hours, and sterile water added if necessary. 100 µL of water remains in Petri dish was transferred and spread on LB agar plates to check for sterility. After the germination (40-48 h), seedlings were transferred in to the sterile Falcon tubes, filled with sterile water (50% distilled water and 50% tap water, v/v) covering only the emerging maize root. Seedlings were placed in a 16-hour light/8-hour dark plant growth chamber, 24°C. For the first two days, the level of sterile water was compensated up to the tip of the maize corn.

Collection of root exudates began from the third day, and took place every second day. The collection process was repeated five times. Collected exudates were pooled together, stored at -80°C and freeze-dried. The lyophilized exudates were weighted and dissolved in distilled water to obtain end concentration of 5 mg/ml. After centrifugation, supernatant was filtered and stored in sterile tubes at -80°C in dark until use.

4.3 Protein extraction

4.3.1 Cytoplasmic protein fraction

Bacteria were harvested at OD_{600} 3.0 what corresponded to the transition phase, by centrifugation (6000 r.p.m., 4°C, 10 min) and washed twice with ice-cold 100 mM Tris (HCl pH 7.7). Cells were resuspended in TE buffer (10 mM Tris, pH 7.5; 1 mM EDTA) with 1 mM PMSF and disrupted by passage through a French press (900 psi). The cell lysate was cleared twice by centrifugation (15000 r.p.m. and 4°C for 30 min); between centrifugations cell lysate was transferred in to a new tube. An aliquot of the supernatant was used for determination of protein concentration with commercially available RotiQuant, and the remainder was stored at -20°C.

4.3.2 Extracellular protein fraction

FZB42 strain cultures were stopped at OD_{600} 3.0 and 4.5 for transition and stationary growth phases. The cells were removed by centrifugation at (5000 r.p.m., 4°C, and 10 min). TCA was added to the liquid fraction to a final concentration of 10% (w/v). The extracellular proteins were precipitated at 4°C overnight. Subsequently, extracellular proteins were collected by centrifugation (12000 r.p.m., 4°C, and 45 min). The protein pellet was washed three times with 2 mL of ice-cold acetone and centrifuged (14000 r.p.m., 4°C, and 20 min). Similarly, two washing steps with 96% ice-cold ethanol were performed. Such obtained extracellular proteins were store in 96% ethanol at -20°C until necessary.

4.4 Two-dimensional gel electrophoresis and protein visualization

For the first dimension of cytosolic proteins, commercially available 18 cm IPG strips in the pH range 4–7 (Amersham Biosciences) were used. Altogether, 500 µg of proteins extract was diluted into 300 µl of rehydration buffer containing 8 M urea, 2 M thiourea, 1% w/v CHAPS, 20 mM DTT and 0.5% v/v Pharmalyte 3–10 (Amersham Biosciences).

For the first dimension of extracellular proteins fraction 24 cm IPG strips in the pH range 3-10 (BioRad) were used. Similarly to the cytosolic proteins 500 µg of extracellular proteins extract was added to the rehydration buffer, which was slightly different from the one mentioned above. The components were as follow: 8 M urea, 2% w/v CHAPS, 50 mM DTT, 0.2% v/v Biolyte Pharmalyte (40%), 3–10 (BioRad)

Proteins extracts dissolved in rehydration buffer were 10 min centrifuged at room temperature, 5000 r.p.m.. Such prepared mixtures were used to re-swell IPG-strip gels, overnight at room temperature.

IEF was performed with IEFCell (Bio-Rad) apparatus for a total employing the following voltage profile: linear increase from 0 to 500 V for 500 Vh, 500 V for 2500 Vh, linear increase from 500 to 3500 V for 10 000 Vh, and a final phase of 3500 V for 35 000 Vh.

After the IEF, the IPG strips were incubated for 15 min in equilibration buffer (6M urea, 30% v/v glycerol, 50mM Tris-HCl (pH 6.8), 4% v/v SDS) containing 0.35% w/v DTT. Subsequently, the IPG strips were transferred into the same equilibration buffer containing 4.5% w/v iodoacetamide instead of DTT, and incubated for another 15 min.

For the second dimension 12.5% and 15% polyacrylamide gels for cytosolic and extracellular proteins, respectively, were precasted. IPG-strips were placed on the top of the polyacrylamide gels and covered by 0.5% agarose with addition of bromophenol blue. Second dimension was carried out at 16°C with an initial starting current of 3 mA/gel for 2 h, followed by main run at 20 mA/gel. The run was continued until the bromophenol blue dye had run off the end of the gel. The resulting 2D gels were fixed with 50% v/v ethanol, 12% v/v acetic acid, for 2 hours followed by two washing steps, and subsequently stained with colloidal Coomassie blue staining (CCB) solution (10% w/v $(NH4)_2SO_4$, 10% v/v phosphoric acid, 20% v/v methanol, 1.2g/L Coomassie blue G250). After about 20 hours of staining, the excess Coomassie was removed by washing the gels in distilled water.

4.5 Image analysis

Commassie stained gels were digitalized by scanning with a Molecular Imager FX scanner (BioRad). Obtained images of cytosolic proteins were analyzed by REDFIN software from Ludesi (Uppsala, Sweden). The analysis consisted in spot detection, MW and p*I* calculation, and quantitative expression analysis. The shapes of detected spots were reviewed, and manually edited if necessary. Normalization was performed by percentage calculation of intensity of each spot related to the total spot intensity per gel.

2-DE gels of extracytoplasmic proteins were analyzed with PDQest software (version 7.2, Bio-Rad). The analysis consisted in spot detection, matching, creation of average-gel, MW and p*I* calculation, and quantitative expression analysis. The gel images were optimized by removing background spots. For normalization ready formula was used which divides the raw quantity of each spot in a gel by the total intensity value of all the pixels in the image. Later the spots were quantified by percentage calculation of intensity of each spot related to the total spot intensity per gel.

For the creation of cytosolic and extracellular reference maps, a protein was considered as expressed under applied conditions, only if was detected in two out of three biological replicates. For each biological replicate, three technical replicates were done.

4.6 Differential expression of proteins

Expression ratios of proteins were calculated base on the numerical data exported to the Excel from Ludesi or PDQest software. Following formula was applied to determine differentially expressed proteins of FZB42 in the presence of root exudates: for up-regulated, fold change= (WT+ Root Exudates /

WT - Root Exudates), and for down-regulated, fold change = - *(WT + Root Exudates / WT - Root Exudates). Proteins were considered as differentially expressed if the ratio was <1.5 and > -1.5, p> 0.05, and if similar trend of up or down-regulation was observed in at least two out of three biological replicates with three technical replicates (n=18).

To determine the involvement of global regulators and chosen sigma factors, gels of wild type were compared with gels of mutants, both obtained from the cultures grown in the presence of root exudates. In case of tested mutants, three biological replicates, with one technical replicate were done.

4.7 Identification of protein spots by MALDI TOF-MS

To identify visualized proteins, spots were excised either manually or with the Proteome WorksTM Spot Cutter System (Bio-Rad, Hercules, CA, USA). In-gel trypsin digestion of the proteins and extraction of the peptides were done in the Ettan Spot Handling Workstation (Amersham Biosciences). Obtained peptide masses were measured in a Voyager-DE STR or in a Proteomics Analyzer 4700 (both Applied Biosystems). Resulting peptide mass fingerprints were analyzed using the gpmaw software. Some proteins were identified with tandem mass spectrometry. To analyze MS/MS data the BioanalystTM Software (Applied Biosystems) and the integrated Mascot (Matrix Science Ltd) script were employed.

4.8 Software and bioinformatics approaches

The protein sequences of *B. amyloliquefaciens* FZB42 were derived from Genebank database. The prediction of proteins' subcellular localization was done with PrediSi software. The signal peptide sequences were searched with SignalP version 3.0, TatP version 1.0 and SecretomeP version 2.0, available at CBS prediction server http://www.cbs.dtu.dk./services/ (Nielsen *et al*. 1997; Bendtsen *et al*. 2005). The transmembrane helixes of the proteins were predicted with TMHMM program, version 2.0 (Krogh *et al*. 2001). For preparation of theoretical proteome of B. *amyloliquefaciens*, JVirGel software was employed (Hiller *et al*. 2003). The observed MW and p*I* outliers were calculated with MS Excel (Microsoft), for both linear functions were applied. Codon Adaptation Index (CAI) was calculated with codonW (http://www.molbiol.ox.ac.uk/cu/) tool in two steps. First, the indices were calculated with values settled for *B. subtilis*. After verification, that higher values corresponded to highly expressed proteins, evidenced by 2D gels, the CAI values for *B. amyloliquefaciens* genes were calculated. The same software was used for the calculation of the grand average of hydropathy (GRAVY value) of each protein. Reconstruction of the metabolic pathways employed two databases: Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa 2002; Aoki-Kinoshita and Kanehisa 2007) and the Pathway Tools software (Karp *et al*. 2002; Caspi *et al*. 2008).

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Acknowledgments

Firstly, I would like to thank Prof. Rainer Borriss for privilege to join his group and carry out my PhD. I greatly appreciate that he was believing in my potential, and encouraged me to explore science at his laboratory.

I would like to thank dr. Birgit Voight for numerous MALDI TOF analysis and for fruitful discussions and patience for answering my questions.

I am grateful to all my colleges from 'Bakteriengenetik' group for pleasant atmosphere during the working time and precious support. Special thanks to Christiane Müller for being so organized and efficient in the lab management. Special thanks to Xiao-Hua for competent support and assist during my work. Thanks to Oliwia for giving many practical tips and being always willing to help. Special thanks to Romy, for helping me with the German translation of Summary. I would like to especially thank to my work colleagues and friends Eva and Lilia for the scientific and emotional support that you gave me. You definitely made the world more colorful. Thanks to Arul for sharing his strains and to Ben for his discussions. Thanks to other group members: Anto, Romy, Svetlana, Kristin and Tom for being great colleagues. Thanks to Paul for correcting my thesis.

Finally, I would like to thank to my Parents. I am indebted to my Parents, for being a examples of principle, and for letting me explore the life in my own way, and for enormous support that they have given to me. Thanks to my sister Dominika for bringing so much happiness and joy to my life. Thanks to my big brothers for being a great examples of persistence on the way to the target.

At last I would like to thank to my family, Michal and my little daughter Zosia for always being there for me.

List of publications

Kinga Kierul, Birgit Voigt, Carvalhais Lilia, Rainer Borriss."Comprehensive proteome profiling of the plant growth-promoting bacterium *Bacillus amyloliquefaciens* FZB42 in response to maize (*Zea mays*) root exudates". In preparation.

Kinga Kierul, Birgit Voigt, Xiao Hua Chen, Rainer Borriss. "Secretome profiling of the plant growth-promoting bacterium *Bacillus amyloliquefaciens* FZB42 and its response to maize (*Zea Mays*) root exudates". In preparation.

Lilia C. Carvalhais, Paul G. Dennis, Dmitriy Fedoseyenko, Oliwia Makarewicz, **Kinga Kierul**, Nicolaus von Wirén, Rainer Borriss. "Linking Plant Nutritional Status to Plant-Microbe Interactions". PLoS ONE 8(7): e68555. doi:10.1371/journal.pone.0068555

Declaration of autonomy

I hereby declare that the submitted work has been completed by me, the undersigned, and that I have neither used any other than permitted reference sources or materials nor engaged in any plagiarism. All references and other sources used by me have been appropriately acknowledged in the work. I further declare that the work has not been previously submitted for the purpose of academic examination, either in its original or similar form, anywhere else.

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Kinga Kierul

6 Appendix

Table 1 *Bacillus amyloliquefaciens* FZB42 extracellular proteins identified on the 2 DE maps with export signal according to the SignalP and LipoP prediction.

	Protein	ID number	Function/similarity	SPase	Localization [d]	% abundance [e]		MW (kDa)	pI	Matched peptides
						ex	st			
	Cell wall									
1	CwlO	RBAM032020	Hypothetical protein	SPase I	Extracellular	3.848	x	50.627	9.02	8
2	DacC	RBAM018470	Penicillin-binding protein	SPase I	Unknown	x	0.257	53.186	8.87	20
3	PonA	RBAM020470	Penicillin-binding proteins IA/IB	SPase I	Extracellular	0.220	0.490	96.161	4.82	19
4	PbpC	RBAM004370	Penicillin-binding protein 3	SPase II	C M	<0.01	0.140	74.097	7.98	17
5	YocH [a]	RBAM018960	Putative cell-wall binding protein	SPase I	Cell wall	0.560	x	30.743	7.75	7
6	YodJ	RBAM019410	D-alanyl-D-alanine carboxypeptidase	SPase II	C M	<0.01	x	30.454	8.27	12
	Membrane bioenergetics									
7	QcrA	RBAM020720	Menaquinol-cytochrome c reductase iron-sulfur subunit	SPase I	Unknown	x	<0.01	18.784	8.29	4
	Sporulation									
8	TasA	RBAM022940	Translocation-dependent antimicrobial spore component	SPase I	Unknown	0.947	0.777	28.111	6.34	8
	Transport/binding proteins and lipoproteins									
9	AppA	RBAM011380	Oligopeptide ABC transporter	SPase II	Cell wall	0.353	0.139	61.336	7.03	10
10	FeuA	RBAM002120	Iron-binding protein	SPase II	Unknown	0.277	1.698	35.191	8.25	17
11	OppA	RBAM011430	Oligopeptide ABC transporter (binding protein)	SPase II	Cell wall	0.460	3.358	61.006	8.37	21
12	PstS	RBAM023290	Phosphate ABC transporter (binding protein)	SPase II	Unknown#	x	0.973	31.827	5.78	10
13	PtsG	RBAM013660	Glucose-specific enzyme IICBA component	TMH	C M	0.347	x	75.516	5.72	14
14	RBAM 1187	RBAM011870	Putative ABC-type multidrug transport system, permease	TMH	C M	2.460	1.559	27.566	9.18	12
15	YckK	RBAM003780	Hypothetical protein RBAM003780	SPase II	Unknown	<0.01	x	29.056	9.3	14
16	YclQ	RBAM004080	Putative ferrichrome ABC transporter	SPase II	Unknown	0.114	0.593	34.831	8.7	13
17	YxeB	RBAM036560	Hypothetical protein RBAM036560	SPase II	C M	0.199	<0.01	35.567	9.11	13
	Protein modification									
18	YxaL	RBAM036900	Hypothetical protein RBAM036900	SPase I	Unknown	3.779	3.448	44.115	8.34	8

	RNA modification									
19	RBAM 31940	RBAM031940	Ribonuclease precursor (barnase)	SPase I	Extracellular	x	0.555	16.654	9.43	7
	Metabolism of amino acids and related molecules									
20	AprE	RBAM010500	Serine alkaline protease (subtilisin)	SPase I	Extracellular	0.780	4.738	24.985	6.17	6
21	Bpr	RBAM015130	Bacillopeptidase F	SPase I	Extracellular	0.807	2.110	15.4242	5.77	13
22	NprE	RBAM014550	Extracellular neutral metalloprotease	SPase I	Extracellular	3.465	3.934	56.768	8.47	11
23	Vpr	RBAM035320	Extracellular serine proteas	SPase I	Extracellular	3.724	2.938	85.769	6.81	19
	Metabolism of carbohydrates and related molecules									
24	AbnA	RBAM025870	Arabinan-endo 1.5-alpha-L-arabinase	SPase I	Extracellular	0.479	0.310	34.954	8.89	12
25	AmyE	RBAM003280	Alpha-amylase	SPase I	Extracellular	0.193	2.314	71.648	6.69	22
36	BglC	RBAM018100	Endo-1.4-beta-glucanase	SPase I	Extracellular	x	0.319	55.131	8.39	5
27	BglS	RBAM036190	Endo-beta-1.3-1.4 glucanas	SPase I	Extracellular	1.655	2.622	27.415	6.89	12
28	Csn	RBAM029740	Chitosanase	SPase I	Extracellular	1.858	1.888	31.401	8.35	13
29	GanA	RBAM012120	Predicted arabinogalactan endo-1.4-beta-galactosidase	SPase I	Unknown	2.140	0.990	41.297	8.56	9
30	LicB	RBAM035790	Lichenan-specific enzyme IIB component	SPase I	Cytoplasmic	<0.01	x	10.947	6.71	11
31	Pel	RBAM007720	Pectate lyase	SPase I	Extracellular	2.783	2.042	45.415	8.17	22
32	PelB	RBAM036320	Pectate lyas	SPase I	Extracellular	0.099	0.189	38.389	9.12	12
33	SacB	RBAM037650	Levansucras	SPase I	Extracellular	5.083	3.761	53.012	6.34	12
34	XynA	RBAM033790	Endo-1.4-beta-xylanase	SPase I	Extracellular	x	1.171	23.236	9.5	1
35	XynD	RBAM018150	Endo-1.4-beta-xylanase	SPase I	Extracellular	x	2.222	54.383	7.7	6
36	YdhT	RBAM035930	Hypothetical protein RBAM035930	SPase I	Unknown	0.954	0.657	40.209	5.38	9
37	YnfF	RBAM018140	Conserved hypothetical protein RBAM018140	SPase I	Unknown	1.561	2.211	47.792	8.31	10
38	YxiA	RBAM036390	Hypothetical protein RBAM036390	SPase I	Unknown	x	1.158	51.685	8.14	20
	Metabolism of coenzymes and prosthetic groups									
39	Ggt	RBAM018540	Gamma-glutamyltranspeptidase	SPase I	Extracellular	0.431	3.979	64.314	6.31	13
	Metabolism of lipids									
40	FabF	RBAM011340	Beta-ketoacyl-acyl carrier protein synthase II	SPase I	Cytoplasmic	0.208	0.311	43.685	5.25	16

41	Lip	RBAM003010	Triacylglycerol lipase	SPase I	Unknown	0.663	1.033	22.730	9.82	8
	Metabolism of nucleotides and nucleic acids									
42	RBAM029550	RBAM029550	Hypothetical protein RBAM029550	SPase I	Unknown	0.894	0.513	32.023	5.38	8
43	YfkN[RR]	RBAM008030	Bifunctional 2',3'-cyclic nucleotide 2'-phosphodiesterase/3'-nucleotidase precursor protein	SPase I	Cell wall	0.818	1.774	156.048	6.39	17
44	YhcR	RBAM009450	Putative 5'-nucleotidase	SPase I	Cell wall	1.328	1.239	65.182	5.47	13
45	Bsn [b]	RBAM029600	Putative extracellular ribonuclease precursor	SPase I	Extracellular	0.688	x	32.023	5.38	9
	Metabolism of phosphate									
46	PhoA	RBAM009670	Alkaline phosphatase A	SPase I	Extracellular	x	0.106	49.643	9.52	13
47	Phy	RBAM019640	3-phytase precursor	SPase I	Extracellular	x	2.522	41.697	5.02	11
48	PhoD	RBAM002930	Phosphodiesterase/alkaline phosphatase	RR	Extracellular	x	0.077	65.922	8.94	18
	Detoxification									
49	PenP	RBAM012080	Beta-lactamase precursor	SPase I	Extracellular	<0.01	0.165	33.030	8.93	18
50	Blm	RBAM011860	Beta-lactamase II precursor (penicillinase)	SPase I	Unknown	1.510	x	27.566	9.18	11
	Transposon and IS									
51	YocA	RBAM018900	Conserved hypothetical protein RBAM018900	TMH	Unknown	<0.01	x	25.129	8.17	3
	Similar to unknown proteins from other organisms									
52	RBAM017540	RBAM017540	Putative chitin binding protein	SPase I	Unknown	x	0.802	22.437	7.89	9
53	RBAM030640	RBAM030640	Hypothetical protein RBAM030640	SPase I	Unknown	x	2.701	29.074	8.5	10
	No similarity									
54	RBAM004640	RBAM004640	Hypothetical protein RBAM004640	SPase I	Unknown	<0.01	0.124	21.383	9.79	8
55	RBAM017640	RBAM017640	Hypothetical protein RBAM017640	SPase I	Unknown	<0.01	0.533	15.990	8.6	12
56	RBAM017400[c]	RBAM017400	Hypothetical protein RBAM017400	SPase I	Unknown	0.297	x	14.966	9.25	8
57	RBAM018080	RBAM018080	Hypothetical protein RBAM018080	SPase I	Unknown	x	0.501	10.062	9.08	4
	Similar to unknown proteins from *B. subtilis*									
58	RBAM036150	RBAM036150	Hypothetical protein RBAM036150	SPase I	Unknown	x	0.562	25.444	9.33	13
59	YdhK	RBAM006150	Conserved hypothetical protein RBAM006150	SPase II	Unknown	x	1.469	23.688	9.45	10

60	YolA1	RBAM002540	Hypothetical protein RBAM002540	SPase I	Unknown	x	0.701	16.898	9.33	5
61	YrpD	RBAM010640	Conserved hypothetical protein RBAM010640	SPase I	Unknown	1.177	1.944	25.262	9.39	15
62	YuaB	RBAM028180	Conserved hypothetical protein RBAM028180	SPase I	Unknown	0.518	0.822	19.781	9.39	10
63	YusA	RBAM029810	Hypothetical protein RBAM029810	SPase II	Unknown	<0.01	<0.01	30.180	9.21	6
64	YwoF	RBAM032390	Hypothetical protein RBAM032390	SPase I	Unknown	0.637	3.222	50.851	5.58	18
65	YycO	RBAM037190	Hypothetical protein RBAM037190	SPase I	Unknown	x	1.367	27.073	8.85	10

[a] Extracellular protein identified in *B. amyloliquefaciens* Δ*degU* mutant.

[b] Extracellular protein identified in *B. amyloliquefaciens* Δ*sigD* mutant.

[c] Extracellular protein identified in *B. amyloliquefaciens* Δ*abrB* mutant.

[d] Localization of proteins was predicted with PSort, CM – cytoplasmic membrane. [#] This protein may have multiple localization sites.

ex: exponential growth phase; st: stationary growth phase. x: protein not found.

[e] The percentage of protein abundance corresponds to the percentage of specific spot volume related to the total spot volume in the extracellular proteome. The PDQuest quantification tool calculated the percentage of specific spot volume after the background subtraction.

Table 2 Extracellular proteins of *Bacillus amyloliquefaciens* FZB42 without typical export signal.

	Protein	ID number	Function/similarity	SecretomeP	% abundance		MW		Matched pepti-
					ex	st			
	Germination								
1	YkvT	RBAM013590	Spore cortex-lytic enzyme		x	0.150	23.245	9.85	7
2	YqiG	RBAM022520	Putative NADH-dependent flavin oxidoreductase		0.288	0.111	41.150	5.2	19
	Membrane bioenergetics (electron transport chain and ATP synthase)								
3	TrxA	RBAM025560	Thioredoxin		<0.01	<0.01	11.386	4.5	7
4	YodC	RBAM019310	Putative nitroreductase		<0.01	<0.01	22.419	5.2	7.0
5	AtpA	RBAM033990	Synthase (subunit alpha)		1.183	x	54.689	5.3	18
	Mobility and chemotaxis								
6	FliD	RBAM032500	Flagellar hook-associated protein 2 (HAP2)	NCS	0.207	x	56.468	5.35	15.0
7	FlgE	RBAM016120	Flagellar hook protein	NCS	x	0.216	27.082	4.97	4
8	FlgK	RBAM032560	Flagellar hook-associated protein 1 (HAP1)	#	0.583	0.345	53.925	4.75	17
9	Hag	RBAM032510	Flagellin protein	#	4.533	2.984	29.490	5.11	20
	Sporulation								
10	SpoVG	RBAM000580	Stage V sporulation protein		0.587	0.250	10.858	5.3	8
	Transport/binding proteins and lipoproteins								
11	PtsI	RBAM013680	Phosphoenolpyruvate-protein phosphotransferase		0.341	x	62.93	4.8	20.0
12	YfnI	RBAM007510	Putative phosphoglycerol transferase		x	<0.01	73.66	6.6	18
	Protein folding								
13	GroEL	RBAM006480	Class I heat-shock protein (chaperonin)	NCS	0.388	0.634	57.383	4.8	19
14	GroES	RBAM006470	Class I heat-shock protein (chaperonin)	NCS	0.225	x	10.169	4.8	10.0
15	Tig	RBAM025290	Trigger factor (prolyl isomerase)	NCS	1.307	x	47.385	4.4	19.0
16	DnaK	RBAM023770	Class I heat-shock protein (molecular chaperone)	NCS	1.310	0.887	66.028	4.7	14.0
	Protein synthesis; Elongation								
17	FusA	RBAM001370	Elongation factor G	NCS	1.629	0.914	76.486	4.8	9

18	TufA	RBAM001380	Elongation factor Tu	NCS	<0.01	0.150	43.385	4.8	15.0
	Protein synthesis; Initiation								
19	InfA	RBAM001640	Translation initiation factor IF-I		0.826	0.105	8.208	6.8	8.0
20	YugI	RBAM028490	Putative polyribonucleotide nucleotidyltransferase; general stress protein 13		0.895	<0.01	14.300	5.95	5
	Metabolism of amino acids and related molecules								
21	Ald	RBAM028980	Alanine dehydrogenase		1.155	0.924	39.29	5.4	18
22	Asd	RBAM016590	Aspartate-semialdehyde dehydrogenase		0.973	0.290	37.9	5.4	20
23	CysK	RBAM000840	Cysteine synthetase A		0.396	0.170	32.77	5.3	13
24	GlnA	RBAM017260	Glutamate-ammonia ligase	NCS	x	<0.01	50.265	5.0	15
25	IlvD	RBAM020010	Dihydroxy-acid dehydratase		0.071	x	59.366	5.3	19.0
26	PepT	RBAM036090	Peptidase T (tripeptidase)		<0.01	<0.01	45.582	4.9	20
27	RocA	RBAM034980	3-hydroxy-1-pyrroline-5 carboxylate dehydrogenase	NCS	0.530	<0.01	56.335	6.0	34.0
28	RocD	RBAM037250	Ornithine aminotransferas	NCS	<0.01	x	43.702	5.3	14.0
29	RocF	RBAM037230	Arginase	NCS	<0.01	<0.01	32.152	4.9	16.0
30	YcgN	RBAM003450	1-pyrroline-5-carboxylate dehydrogenase like protein		0.485	1.105	56.38	5.4	19
	Metabolism of carbohydrates and related molecules								
31	Eno	RBAM031260	Enolase	NCS	0.805	0.458	46.588	4.7	25.0
32	FbaA	RBAM034270	Putative translaldolase		1.747	0.424	30.37	5.2	11
33	GapA	RBAM031300	Glyceraldehyde-3-phosphate dehydrogenase	NCS	0.268	0.379	35.761	5.4	16.0
34	Mdh	RBAM026160	Malate dehydrogenase	NCS	1.208	1.483	33.546	5.06	11.0
35	PdhA	RBAM014420	Pyruvate dehydrogenase (E1 alpha subunit)	NCS	2.303	x	41.337	6.2	15.0
36	PdhB	RBAM014430	Pyruvate dehydrogenase (E1 beta subunit)	NCS	0.184	0.377	35.505	4.8	29.0
37	PdhC	RBAM014440	Pyruvate dehydrogenase (dihydrolipoamide acetyltransferase E2 subunit)	NCS	0.695	0.210	47.563	5.2	19.0
38	PdhD	RBAM014450	Dihydrolipoamide dehydrogenase (E3 subunit)	NCS	0.908	0.871	26.638	8.7	14.0
39	Pgm	RBAM012200	2,3-bisphosphoglycerate-independent phosphoglycerate mutase		1.491	1.024	56.221	5.3	21

40	AlsS	RBAM033170	Acetolactate synthase		1.345	<0.01	61.954	5.2	24
41	YdjL	RBAM006650	Putative dehydrogenase		0.076	x	37.38	5.1	14.0
42	IolS	RBAM036790	Inositol utilization protein S		0.168	<0.01	34.79	5.2	13
43	Pta	RBAM034850	Phosphotransacetylase		1.186	0.592	34.74	4.8	15
44	PtsH	RBAM013670	Phosphocarrier protein HPr component		0.183	3.200	9.18	4.86	7
	Metabolism of lipids								
45	Bcd	RBAM022360	Leucine dehydrogenase		0.781	0.703	39.663	5.1	13
46	FabI	RBAM011730	Enoyl-[acyl-carrier-protein] reductase		0.406	<0.01	27.833	5.8	14
	Metabolism of nucleotides and nucleic acids								
47	Adk	RBAM001620	Adenylate kinase		1.421	0.164	24.09	4.8	9
	Adaptation to atypical conditions								
48	CspB	RBAM009370	Major cold-shock protein		1.301	0.297	7.25	4.6	4
49	CspC	RBAM005400	Cold-shock protein		2.340	0.277	7.365	4.7	7.0
50	CspD	RBAM020070	Cold-shock protein		1.599	0.303	7.318	4.5	4.0
	Detoxification								
51	KatA	RBAM009090	Vegetative catalase	NCS	<0.01	<0.01	51.042	8.6	20
52	SodA	RBAM023340	Superoxide dismutase [Mn]	NCS	1.711	1.326	22.365	5.2	11.0
53	Tpx	RBAM026420	Thiol peroxidase		0.345S	<0.01	18.148	5.0	13.0
54	YceD	RBAM003160	Putative tellurium resistance protein	NCS	<0.01	x	20.694	4.5	2.0
55	YceE	RBAM003170	Putative tellurium resistance protein	NCS	<0.01	x	20.867	4.6	6.0
56	AhpC	RBAM036960	Alkyl hydroperoxide reductase subunit C (small subunit)		1.800	<0.01	20.512	4.5	7
	Similar to unknown proteins from *B. subtilis*								
57	YflE	RBAM007920	Conserved hypothetical protein RBAM007920		2.275	0.830	73.87	6.2	14
58	YhgC	RBAM010330	Hypothetical protein RBAM010330		<0.01	<0.01	18.70	5.6	4
59	YurX	RBAM029780	Conserved hypothetical protein RBAM029780		0.606	0.483	48.26	5.3	16
60	YxkC	RBAM 036070	Hypothetical protein RBAM36070		<0.01	x	22.779	9.46	10

ex: exponential growth phase.

st: stationary growth phase.

x: protein not found.

NCS proteins secreted via non-classical pathway identified by SecretomeP.

protein known to be secreted in other bacteria.

Table 3 Cytosolic proteins isolated from *B. amyloliquefaciens* strain FZB42. Cells were harvested at transition growth phase, proteins were identified by MALDI-MS and MS/MS on the 2-D gel of analytical window p*I* 4-7.

	Gene	ID	Protein function / similarity	MW theor.[a]	MW obser.[b]	pI calc.[c]	pI obser.[d]	MM[e]	Cellular localization[f]	CAI[g]	GRAVY[h]	Peptide count
	Cell envelope and cellular processes; Cell division (4 different proteins)											
1	*divIVA*	RBAM015250	cell-division initiation protein	19.30	19.1	5.06	5.07	+	C	0.62	-0.799	9
2	*gidA*	RBAM038100	glucose inhibited division protein A	69.63	74.75	5.97	6.14	+	C	0.57	-0.4	11
3	*ftsZ*	RBAM015120	cell-division initiation protein	40.29	36.16	4.91	4.37		U	0.64	-0.199	16
4	*gid*	RBAM015960	glucose-inhibited division protein	48.23	53.17	5.69	6.09		U	0.59	-0.287	20
	Cell envelope and cellular processes; Cell wall (12 different proteins)											
5	*yabE*	RBAM000490	conserved hypothetical protein	49.70	56.83	9.48	6.79		SecP	0.55	-0.411	10
6	*dacA*	RBAM000130	serine-type D-ala-D-ala carboxypeptidase	48.63	51.28	6.41	6.4		SpI	0.65	-0.332	13
7	*gtaB*	RBAM032820	UTP-glucose-1-phosphate uridylyltransferase	33.29	31.82	5.11	5.14	+	C	0.69	-0.409	15
8	*ddl*	RBAM004900	D-alanyl-D-alanine ligase	39.19	42.81	4.78	4.78		C	0.59	-0.256	18
9	*mreB*	RBAM025080	rod shape-determining protein	35.77	35.03	5.07	5.11	+	C	0.66	0.088	19
10	*mbl*	RBAM033610	mreB-like protein	35.85	34.49	5.77	6.07		C	0.62	0.068	20
11	*murAB*	RBAM034260	UDP-N-acetylglucosamine 1-carboxyvinyltransferase	46.00	51.49	5.99	6.68		C	0.48	0.013	14
12	*murB*	RBAM015090	UDP-N-acetylenolpyruvoylglucosamine reductase	32.99	32.07	5.56	5.89		C	0.57	-0.238	19
13	*murE*	RBAM015040	UDP-N-acetylmuramoylalanyl-D-glutamate-2,6-diaminopimelate ligase	53.91	54.99	5.52	5.88	+	C	0.57	-0.263	26
14	*murC*	RBAM026910	UDP-N-acetyl muramate-alanine ligase	48.35	47.89	5.26	5.26	+	C	0.61	-0.175	23
15	*RBAM032860*	RBAM032860	minor teichoic acid biosynthesis protein ggaA like protein	59.89	59.33	6.2	6.68	+	C	0.57	-0.526	16
16	*murAA*	RBAM033910	UDP-N-acetylglucosamine 1-carboxyvinyltransferase	46.62	52.7	5.49	5.67	+	C	0.69	0.027	10

	Cell envelope and cellular processes; Membrane bioenergetics (electron transport chain and ATP synthase) (12 different proteins)											
17	*yjlD*	RBAM012370	NADH dehydrogenase-like protein	41.94	38.41	6.69	5.21	+	C	0.76	-0.023	13
18	*yqiG*	RBAM022520	putative NADH-dependent flavin oxidoreductase	41.15	49.91	5.15	5.19		C	0.59	-0.363	10
19	*atpD*	RBAM033960	ATP synthase (subunit beta)	51.39	54.06	4.87	4.79	+	C	0.68	-0.115	15
20	*atpA*	RBAM033980	ATP synthase (subunit alpha)	54.69	54.98	5.34	5.39	+	C	0.69	-0.124	18
21	*yfmJ*	RBAM007690	putative NADP-dependent oxidoreductase	36.75	36.07	4.83	4.76		C	0.54	-0.242	10
22	*yfkO*	RBAM008000	putative NAD(P)H nitroreductase	25.64	24.85	5.71	5.94		C	0.62	-0.552	13
23	*yojN*	RBAM019150	putative nitric-oxide reductase	32.55	34.47	4.94	4.87		C	0.59	-0.136	4
24	*etfA*	RBAM025580	electron transfer flavoprotein (alpha subunit)	34.00	32.03	4.68	4.54		C	0.57	0.067	13
25	*etfB*	RBAM025590	electron transfer flavoprotein (beta subunit)	28.27	31.32	4.38	4.54		C	0.55	-0.287	9
26	*yumC*	RBAM029160	conserved hypothetical protein	36.64	39.84	5.25	5.27	+	C	0.62	-0.201	18
27	*yutJ*	RBAM029310	putative NADH dehydrogenase	39.41	46.31	6.14	6.75		U	0.58	-0.312	12
28	*yodC*	RBAM019310	putative nitroreductase	22.42	23.1	5.15	5.2	+	C	0.67	-0.151	8
	Cell envelope and cellular processes; Mobility and chemotaxis (2 different proteins)											
29	*hag*	RBAM032510	flagellin protein	29.49	29.02	5.11	5.08	+	SecP	0.79	-0.456	18
30	*fliY*	RBAM016160	flagellar motor switch protein	40.93	55.79	4.38	4.38		C	0.58	-0.249	15
	Cell envelope and cellular processes; Protein secretion (1 protein)											
31	*secA*	RBAM032450	translocase binding subunit (ATPase)	95.23	92.83	5.32	5.39	+	C	0.59	-0.544	14
	Cell envelope and cellular processes; Sensors (signal transduction) (2 different proteins)			0.00								
32	*cheA*	RBAM016270	two-component sensor histidine kinase, Chemotaxis protein	74.40	83.44	4.73	4.67		C	0.57	-0.218	10
33	*luxS*	RBAM027680	s-ribosylhomocysteine lyase	17.68	19.3	5.27	5.34	+	C	0.64	-0.254	6
	Cell envelope and cellular processes; Sporulation (6 different proteins)											
34	*tasA*	RBAM022940	spore coat-associated protein N	28.11	28.61	6.34	5.68	+	SpI	0.64	-0.476	15
35	*spoVG*	RBAM000580	stage V sporulation protein	10.86	13.3	5.25	5.38		C	0.64	-0.497	8
36	*kipI*	RBAM004310	sporulation inhibitor	26.74	27.9	5.5	5.71		C	0.51	-0.145	6

37	*obgE*	RBAM024970	spo0B associated GTP-binding protein	47.63	55.71	5.01	5.08		C	0.58	-0.435	20
38	*spsC1*	RBAM031230	spore coat polysaccharide biosynthesis protein	41.60	41.85	5.93	6.41		C	0.60	-0.12	11
39	*kipA*	RBAM004320	kipI antagonist	36.63	38.53	5.68	5.06		C	0.47	-0.059	5
	Cell envelope and cellular processes; Transformation/competence (2 different proteins)											
40	*mecA*	RBAM011520	adapter protein	25.95	28.34	4.39	4.24		C	0.59	-0.746	11
41	*cinA*	RBAM016770	competence-damage inducible protein	44.98	48.02	5.34	5.21		C	0.53	-0.166	16
	Cell envelope and cellular processes; Transport/binding proteins and lipoproteins (11 different proteins)											
42	*oppA*	RBAM011430	oligopeptide ABC transporter (binding protein)	61.01	55.68	8.37	6.79		SpII	0.65	-0.623	7
43	*yufN*	RBAM028640	hypothetical lipoprotein	38.01	34.06	6.86	5.52		SpII	0.61	-0.379	13
44	*yccK*	RBAM003030	conserved hypothetical	34.56	34.56	5.33	5.39		C	0.49	-0.378	15
45	*ptsI*	RBAM013680	phosphoenolpyruvate-protein phosphotransferase	62.93	66.03	4.81	4.85	+	C	0.68	-0.128	20
46	*yhaQ*	RBAM010130	putative ABC transporter (ATP-binding protein)	33.60	30.9	6.69	6.88		CM	0.49	-0.278	9
47	*ecsA*	RBAM010280	ABC-type transporter ATP-binding protein	27.71	29.56	5.72	6.04		CM	0.52	-0.171	10
48	*appD*	RBAM011360	oligopeptide transport ATP-binding protein	36.06	33.86	5.54	5.73		CM	0.52	-0.128	16
49	*ykpA*	RBAM014170	ABC transporter (ATP-binding protein)	60.82	63.89	5.19	5.11	+	CM	0.64	-0.316	14
50	*oppD*	RBAM011460	oligopeptide ABC transporter (ATP-binding protein)	39.67	46.29	6.28	6.76		CM	0.54	-0.211	23
51	*yufO*	RBAM028650	putative ABC transport system, ATPase component	56.39	56.89	5.93	6.36		CM	0.53	-0.259	22
52	*yurY*	RBAM029790	putative ABC transporter (ATP-binding protein)	29.04	28.04	4.81	4.74	+	CM	0.72	-0.277	16
	Information pathways; DNA recombination (3 different proteins)											
53	*gyrA*	RBAM000070	DNA gyrase subunit A	88.70	100.3	5.46	5.69		C	0.60	-0.353	14
54	*recA*	RBAM016780	multifunctional SOS repair regulator	37.93	40.93	5	5.09	+	C	0.63	-0.341	21
55	*gyrB*	RBAM000060	DNA topoisomerase (ATP-hydrolyzing) chain B	71.50	73.88	5.43	5.67		C	0.59	-0.513	12
	Information pathways; DNA replication (1 protein)			0.00								
56	*dnaA*	RBAM000010	chromosomal replication initiatior protein	50.81	56.04	6.01	6.59	+	C	0.55	-0.401	19
	Information pathways; DNA restriction/modification and repair (4 different proteins)											
57	*RBAM007140*	RBAM007140	type I restriction-modification system methyltransferase subunit like protein	60.22	57.11	5.31	5.41		SecP	0.61	-0.543	17

58	*ypvA*	RBAM020280	probable ATP-dependent helicase	73.70	67.73	4.78	4.73		C	0.54	-0.41	14
59	*uvrB*	RBAM032310	UvrABC system protein B	76.42	73.78	5.36	5.47		C	0.51	-0.507	26
60	*mutS*	RBAM016880	DNA mismatch repair protein	97.88	98.65	5.44	5.63		C	0.57	-0.384	13
	Information pathways; Protein folding (4 different proteins)											
61	*groES*	RBAM006470	class I heat-shock protein (chaperonin)	10.17	7.71	4.8	4.69		C	0.74	-0.118	10
62	*groEL*	RBAM006480	class I heat-shock protein (chaperonin)	57.38	58.04	4.75	4.68	+	C	0.76	-0.055	24
63	*dnaK*	RBAM023770	class I heat-shock protein (molecular chaperone)	66.03	71.89	4.73	4.69	+	C	0.75	-0.413	14
64	*tig*	RBAM025290	trigger factor (prolyl isomerase)	47.39	56.82	4.42	4.31	+	C	0.79	-0.561	15
	Information pathways; Protein modification (6 different proteins)											
65	*mlpA*	RBAM016550	putative zinc protease	45.93	49.41	5.16	5.19		SecP	0.56	-0.441	12
66	*ppiB*	RBAM021460	peptidyl-prolyl cis-trans isomerase B	15.37	16.2	5.87	6.13		C	0.75	-0.557	4
67	*yqhT*	RBAM022790	putative dipeptidase	38.36	38.47	5.02	4.93		C	0.53	-0.089	16
68	*ytjP*	RBAM027080	putative peptidase	50.82	55.82	4.99	4.94		C	0.58	-0.39	12
69	*yuiE*	RBAM029100	putative leucyl aminopeptidase	53.34	55.68	4.88	5		C	0.53	-0.193	21
70	*hprK*	RBAM032190	HPr kinase/phosphorylase (HPrK/P) (HPr(Ser) kinase/ phosphorylase)	34.66	34.67	5.23	5.36		C	0.49	-0.196	14
	Information pathways; Protein synthesis; Aminoacyl-tRNA synthetases (22 different proteins)											
71	*argS*	RBAM034470	arginyl-tRNA synthetase (Arginine--tRNA ligase)	62.54	57.91	5.34	5.36	+	C	0.66	-0.444	12
72	*ytpR*	RBAM026940	putative phenylalanyl-tRNA synthetase (beta subunit)	21.71	22.9	4.67	4.55		C	0.65	-0.157	13
73	*serS*	RBAM000160	seryl-tRNA synthetase	48.86	54.03	5.3	5.36	+	C	0.63	-0.557	17
74	*metS*	RBAM000470	methionyl-tRNA synthetase	75.91	76.76	5.15	5.21	+	C	0.63	-0.438	21
75	*lysS*	RBAM000920	lysyl-tRNA synthetase	57.53	57.07	5.17	5.21	+	C	0.68	-0.573	23
76	*gltX*	RBAM001170	glutamyl-tRNA synthetase	55.70	57.03	5.2	5.15	+	C	0.63	-0.475	17
77	*cysS*	RBAM001190	cysteinyl-tRNA synthetase	53.74	56.13	5.14	5.19	+	C	0.60	-0.54	13
78	*gatA*	RBAM007080	glutamyl-tRNA(Gln) amidotransferase (subunit A)	52.60	54.96	5.33	5.37		C	0.63	-0.213	20
79	*gatB*	RBAM007090	glutamyl-tRNA(Gln) amidotransferase (subunit B)	53.42	55.99	5.13	5.13	+	C	0.61	-0.472	15
80	*ileS*	RBAM015260	isoleucyl-tRNA synthetase	104.8	98.77	5.36	5.49	+	C	0.67	-0.46	11

81	*proS*	RBAM016410	prolyl-tRNA synthetase	63.08	66.07	5.08	5.13	+	C	0.62	-0.386	23
82	*asnS*	RBAM020510	asparaginyl-tRNA synthetase (Asparagine--tRNA ligase)	49.08	56.02	5.22	5.24	+	C	0.69	-0.35	13
83	*glyS*	RBAM023560	glycine-tRNA ligase beta chain	76.31	73.89	5.44	5.69	+	C	0.60	-0.364	12
84	*glyQ*	RBAM023570	glycyl-tRNA synthetase (alpha subunit)	34.12	30.72	4.96	4.88		C	0.59	-0.444	12
85	*alaS*	RBAM024510	alanyl-tRNA synthetase	96.86	92.67	5.23	5.26	+	C	0.62	-0.36	19
86	*aspS*	RBAM024660	aspartyl-tRNA synthetase	66.02	66.02	4.89	4.9	+	C	0.61	-0.235	31
87	*pheT*	RBAM025700	phenylalanyl-tRNA synthetase (beta subunit)	87.75	91.32	5.19	5.19	+	C	0.59	-0.165	25
88	*pheS*	RBAM025710	phenylalanyl-tRNA synthetase (alpha subunit)	38.91	39.01	5.58	5.89	+	C	0.60	-0.427	15
89	*thrS*	RBAM025990	threonyl-tRNA synthetase	73.70	72.54	5.39	5.52	+	C	0.63	-0.504	27
90	*tyrS*	RBAM026790	tyrosyl-tRNA synthetase	47.73	49.59	5.37	5.31	+	C	0.63	-0.423	13
91	*leuS*	RBAM027250	leucyl-tRNA synthetase	91.21	95.12	4.96	4.92	+	C	0.61	-0.472	15
92	*valS*	RBAM025140	valyl-tRNA synthetase	101.6	97.14	5.19	5.19	+	C	0.61	-0.542	28
	Information pathways; Protein synthesis; Elongation (6 different proteins)											
93	*fusA*	RBAM001370	elongation factor G	76.49	75.65	4.8	4.79	+	C	0.79	-0.283	23
94	*tufA*	RBAM001380	elongation factor Tu	43.39	52.54	4.84	4.76	+	C	0.86	-0.283	19
95	*ylaG*	RBAM014630	putative GTP-binding elongation factor	68.42	75.44	5.19	5.27	+	C	0.65	-0.368	19
96	*tsf*	RBAM016340	translation elongation factor ef-ts	32.29	33.47	5.24	5.31	+	C	0.83	-0.446	18
97	*efp*	RBAM022780	elongation factor P	20.53	20.5	5.06	5.19		C	0.70	-0.334	5
98	*lepA*	RBAM023810	GTP-binding protein	68.42	66.04	5.29	5.18		C	0.57	-0.233	21
	Information pathways; Protein synthesis; Initiation (one protein)											
99	*infB*	RBAM016470	initiation factor (IF-2) InfB	78.55	75.21	5.44	4.4		C	0.65	-0.406	8
	Information pathways; Protein synthesis; Ribosomal proteins (15 different proteins)											
100	*rpsJ*	RBAM001400	ribosomal protein S10 (BS13)	11.66	7.01	9.79	4.15		C	0.78	-0.46	8
101	*rplQ*	RBAM001690	ribosomal protein L17 (BL15)	13.76	14.4	9.9	6.81		C	0.79	-0.731	8
102	*rpsD*	RBAM026590	30S ribosomal protein S4	22.88	23.01	9.88	5.72		C	0.81	-0.651	8
103	*rpsF*	RBAM038000	30S ribosomal protein S6 (BS9)	11.06	7.65	5.25	5.1		C	0.80	-0.505	9
104	*rplK*	RBAM001270	ribosomal protein L11 (BL11)	14.92	14.4	9.3	6.81		C	0.74	-0.06	3

105	*rplJ*	RBAM001290	ribosomal proteinL10 (BL5)	17.99	18.79	5.23	5.18	+	C	0.81	-0.12	18
106	*rplE*	RBAM001530	ribosomal protein L5 (BL6)	20.15	19.4	9.54	6.6		C	0.76	-0.273	14
107	*rpsH*	RBAM001550	ribosomal protein S8 (BS8)	14.85	14	9.86	6.79	+	C	0.80	-0.227	9
108	*rplF*	RBAM001560	ribosomal protein L6 (BL8)	19.43	19.99	9.6	4.55		C	0.80	-0.463	15
109	*rpsE*	RBAM001580	ribosomal protein S5	17.87	17.09	9.92	6.6	+	C	0.80	0.037	11
110	*rpsB*	RBAM016330	ribosomal protein S2 RpsB	27.91	32.1	6.27	6.74	+	C	0.81	-0.47	9
111	*rpmD*	RBAM001590	ribosomal protein L30 (BL27)	6.63	7.4	10.27	6.8		U	0.73	-0.393	5
112	*rplR*	RBAM001570	ribosomal protein L18	12.92	7.3	10.11	6.8		U	0.76	-0.413	5
113	*rplP*	RBAM001480	ribosomal protein L16	16.20	17.6	10.34	4.56		C	0.78	-0.538	3
114	*rplL*	RBAM001300	ribosomal protein L12 (BL9)	12.74	13	4.56	4.38	+	U	0.85	0.115	8
	Information pathways; Protein synthesis; Termination (2 different proteins)											
115	*frr*	RBAM016360	ribosome recycling factor	20.64	20.9	5.33	5.3		C	0.70	-0.538	12
116	*prfB*	RBAM032440	peptide chain release factor II	37.64	51.58	5.21	5.08		C	0.57	-0.728	11
	Information pathways; RNA modification (3 different proteins)											
117	*rph*	RBAM025440	ribonuclease PH	26.70	29.85	5.17	5.23		C	0.58	-0.176	8
118	*ylyB*	RBAM015290	putative pseudouridylate synthase	34.17	39.72	6.05	6.5		C	0.56	-0.505	14
119	*yugI*	RBAM028490	putative polyribonucleotide nucleotidyltransferase	14.29	14.9	5.95	6.78	+	C	0.82	-0.552	7
	Information pathways; RNA synthesis; Elongation (5 different proteins)											
120	*rpoB*	RBAM001320	RNA polymerase (beta subunit)	133.7	130.4	4.94	4.93	+	C	0.68	-0.418	34
121	*rpoC*	RBAM001330	RNA polymerase (beta subunit)	134.0	71.45	8.69	5.71		C	0.68	-0.311	34
122	*rpoA*	RBAM001680	RNA polymerase (alpha subunit)	34.79	43.65	4.8	4.71	+	C	0.68	-0.307	13
123	*greA*	RBAM024420	transcription elongation factor	17.35	19.01	4.75	4.71		C	0.67	-0.509	12
124	*rpoE*	RBAM034320	DNA-directed RNA polymerase delta subunit	20.71	21	3.88	4.1		C	0.67	-1.033	5
	Information pathways; RNA synthesis; Initiation (3 different proteins)											
125	*sigH*	RBAM001230	RNA polymerase sigma-30 factor SigH	25.42	24.1	5.43	5.61		C	0.55	-0.459	9
126	*rpoD*	RBAM023510	RNA polymerase major sigma-43 factor (sigma-A)	43.16	55.94	4.79	4.75		C	0.68	-0.686	10
127	*yvyD*	RBAM032460	conserved hypothetical protein	21.89	23.02	5.64	5.8		C	0.62	-0.826	8

	Information pathways; RNA synthesis; Regulation (10 different proteins)											
128	*pyrR*	RBAM015300	pyrimidine operon transcriptional attenuator anduracil phosphoribosyltransferase	20.21	23.5	5.22	5.2	+	C	0.48	-0.237	12
129	*lexA*	RBAM017650	negative transcriptional regulator of the SOS regulon	23.06	26.5	5.35	5.57		C	0.55	-0.209	7
130	*hpr*	RBAM010230	protease production regulatory protein Hpr	23.83	22.89	5.26	5.33	+	C	0.56	-0.633	9
131	*codY*	RBAM016000	GTP-sensing transcriptional pleiotropic repressor	29.04	28.03	4.9	5.01	+	C	0.64	-0.169	17
132	*resD*	RBAM021260	two-component response regulator	27.43	29.43	5.75	6.83		C	0.53	-0.383	12
133	*spo0A*	RBAM022550	stage 0 sporulation protein A (Spo0A)	29.61	25.08	5.84	6.21		C	0.56	-0.104	17
134	*msmR*	RBAM027180	transcriptional regulator (LacI family)	37.45	34.37	5.83	6.31		C	0.49	-0.182	22
135	*degU*	RBAM032640	two-component response regulator DegU	25.85	27.45	5.66	6.04	+	C	0.61	-0.251	20
136	*yycF*	RBAM037400	putative two-component response regulator	27.32	29.99	5	4.94		C	0.61	-0.384	7
137	*lacR*	RBAM012190	putative lactose phosphotransferase system repressor protein	28.66	29.01	5.8	6.24		C	0.55	-0.308	18
	Information pathways; RNA synthesis; Termination (2 different proteins)											
138	*nusG*	RBAM001260	transcription antitermination factor	20.10	19.9	5.26	5.34	+	SecP	0.61	-0.44	11
139	*nusA*	RBAM016440	transcription elongation protein	41.90	41.25	4.8	4.73	+	C	0.62	-0.392	26
	Metabolism of amino acids and related molecule (61 different proteins)											
140	*yjbG*	RBAM011540	putative oligopeptidase F	69.77	65.33	5.34	5.31	+	SecP	0.61	-0.566	13
141	*ampS*	RBAM014190	aminopeptidase	45.85	51.53	5.29	5.33		SecP	0.63	-0.521	12
142	*aspB*	RBAM020520	aspartate aminotransferase (Transaminase A)	42.68	42.13	5.77	6.08	+	SpI	0.57	-0.235	16
143	*gabT*	RBAM004150	4-aminobutyrate aminotransferase	43.40	53.03	5.56	6.27		C	0.64	0.087	15
144	*ycgN*	RBAM003450	1-pyrroline-5-carboxylate dehydrogenase	56.38	56.71	5.39	5.52		C	0.67	-0.278	15
145	*yhdR*	RBAM009800	putative aspartate aminotransferase	43.97	41.37	5.42	5.22	+	C	0.53	-0.219	20
146	*tdh*	RBAM016830	L-threonine 3-dehydrogenase Tdh	36.94	38.21	6.46	6.81		C	0.56	0.062	8
147	*hutI*	RBAM036430	imidazolone-5-propionate hydrolase	45.23	42.79	5.51	5.73		C	0.49	-0.118	16
148	*cysK*	RBAM000840	cysteine synthetase A	32.77	30.34	5.28	5.31	+	C	0.62	-0.138	22
149	*RBAM003700*	RBAM003700	aminotransferase	49.07	55.87	5.42	5.67	+	C	0.52	-0.399	12
150	*yxeP*	RBAM003950	conserved hypothetical protein	41.81	45.19	5.26	5.26		C	0.56	-0.248	10

151	*metE*	RBAM013040	cobalamin-independent methionine synthase	86.53	90.04	5.1	5.23	+	C	0.62	-0.371	20
152	*ykuQ*	RBAM013950	putative etrahydrodipicolinate succinylase	24.95	26.56	4.87	4.85		C	0.72	0.065	10
153	*ykuR*	RBAM013960	hippurate hydrolase homolog	41.66	42.02	5.4	6.61		C	0.51	-0.191	9
154	*cysC*	RBAM015430	putative adenylyl-sulfate kinase	22.36	21.8	5.27	5.29	+	C	0.61	-0.586	8
155	*asd*	RBAM016590	aspartate-semialdehyde dehydrogenase	37.85	48.91	5.39	5.61	+	C	0.65	-0.238	15
156	*dapG*	RBAM016600	aspartokinase I (alpha and beta subunits)	42.71	47.32	6.08	6.69	+	C	0.57	0.092	17
157	*ymfH*	RBAM016710	conserved hypothetical protein	49.01	55.82	4.96	4.93		C	0.56	-0.425	16
158	*kbl*	RBAM016840	2-amino-3-ketobutyrate coenzyme A ligase	43.15	46.31	6.16	6.76		C	0.54	-0.165	23
159	*glnA*	RBAM017260	glutamine synthetase	50.27	54.07	5.04	5.07	+	C	0.74	-0.286	13
160	*gltB*	RBAM018610	glutamate synthase [NADPH] small subunit	54.85	55.14	6.5	5.29		C	0.56	-0.494	10
161	*ilvA*	RBAM019920	threonine dehydratase IlvA	46.90	51.52	5.75	6.2		C	0.54	-0.263	9
162	*ypwA*	RBAM020270	putative metallocarboxypeptidase	57.87	56.96	4.87	4.85	+	C	0.59	-0.468	10
163	*dapB*	RBAM020640	dihydrodipicolinate reductase	29.36	28.99	5.31	5.39	+	C	0.61	-0.19	14
164	*aroF*	RBAM020860	chorismate synthase	42.64	52.36	5.95	6.33		C	0.60	-0.34	17
165	*gudB*	RBAM021110	NAD-specific glutamate dehydrogenase	47.02	47.59	5.57	5.91		C	0.58	-0.275	10
166	*serA*	RBAM021220	D-3-phosphoglycerate dehydrogenase	57.03	57.07	5.69	6.09	+	C	0.60	-0.057	17
167	*lysA*	RBAM021480	diaminopimelate decarboxylase	48.45	48.98	5.15	5.19		C	0.57	-0.111	19
168	*gcvPA*	RBAM022880	glycine decarboxylase (subunit I) (glycine cleavage system protein P)	49.14	56.43	6.21	6.81		C	0.54	-0.247	16
169	*gcvT*	RBAM022890	aminomethyltransferase (glycine cleavage system protein T)	40.29	40.87	5.92	5.67		C	0.58	-0.295	19
170	*ilvC*	RBAM025350	ketol-acid reductoisomerase (acetohydroxy-acid isomeroreductase)	37.45	35.06	5.39	5.52	+	C	0.68	-0.19	20
171	*aroA*	RBAM026870	chorismate mutase	39.49	40.81	5.59	5.89	+	C	0.63	-0.215	13
172	*asnB*	RBAM027560	asparagine synthetase	72.45	71.93	5.75	6.01		C	0.64	-0.363	25
173	*metK*	RBAM027570	S-adenosylmethionine synthetase	43.87	54.85	5.14	5.15	+	C	0.70	-0.33	17
174	*ald*	RBAM028980	alanine dehydrogenase	39.29	42.94	5.44	5.71		C	0.62	0.084	22
175	*thrC*	RBAM029360	threonine synthase	37.24	39.03	5.17	5.22	+	C	0.58	-0.039	12
176	*hom*	RBAM029370	homoserine dehydrogenase	47.52	56.26	5.02	5.08	+	C	0.56	-0.103	12

177	*csd*	RBAM029770	cysteine desulfurase	44.75	47.56	5.37	5.56	+	C	0.57	-0.166	20
178	*yusX*	RBAM030080	putative oligoendopeptidase	68.07	66.89	5.19	5.23	+	C	0.50	-0.415	12
179	*glyA*	RBAM034060	serine hydroxymethyltransferase	45.45	41.61	5.8	5.71	+	C	0.68	-0.263	18
180	*ywfG*	RBAM034880	putative aspartate aminotransferase (Transaminase A)	44.61	42.35	5.7	5.79		C	0.51	-0.255	6
181	*hutU*	RBAM036420	urocanase	60.89	58.42	5.8	6.19		C	0.53	-0.275	21
182	*dapA*	RBAM016610	dihydrodipicolinate synthase	30.93	29.87	5.13	5.1	+	U	0.58	0.02	20
183	*glmS*	RBAM002320	l-glutamine-D-fructose-6-phosphate amidotransferase	65.33	61.34	4.98	4.91	+	C	0.68	0.043	26
184	*dat*	RBAM009900	D-alanine aminotransferase Dat	31.45	34.43	4.96	4.93	+	C	0.49	-0.333	14
185	*serC*	RBAM010260	phosphoserine aminotransferase SerC	39.44	38.21	5.84	6.24		C	0.58	-0.301	16
186	*yisK*	RBAM010910	putative 5-oxo-1,2,5-tricarboxilic-3-penten aciddecarboxylase	32.64	34.02	5.7	6.1		C	0.55	-0.217	15
187	*cysH*	RBAM015400	phosphoadenosine phosphosulfate reductase	26.80	27.85	5.66	6.85	+	C	0.60	-0.497	8
188	*metA*	RBAM020050	homoserine O-succinyltransferase	35.30	42.36	5.7	6.13		C	0.53	-0.476	10
189	*hisC*	RBAM020780	histidinol-phosphate aminotransferase	40.32	39.98	4.8	4.55	+	C	0.55	-0.249	12
190	*ansB*	RBAM021700	aspartate ammonia-lyase	66.02	66.02	4.89	4.91		C	0.59	-0.154	34
191	*ilvB*	RBAM025370	acetolactate synthase (acetohydroxy-acid synthase) (large subunit)	65.58	56.99	5.67	5.85	+	C	0.55	-0.151	15
192	*speB*	RBAM034610	agmatinase (Agmatine ureohydrolase) (AUH)	32.33	31.89	5.04	5.03	+	C	0.59	-0.238	15
193	*rocA*	RBAM034980	3-hydroxy-1-pyrroline-5-carboxylate dehydrogenase	56.34	48.05	6.01	6.04		C	0.57	-0.239	23
194	*rocG*	RBAM034990	NAD-specific glutamate dehydrogenase (NAD-GDH)	47.03	47.47	5.85	6.26		C	0.52	-0.179	22
195	*ywaA*	RBAM035750	putative branched-chain-amino-acid aminotransferase	40.33	38.52	5.09	5.07	+	C	0.58	-0.177	13
196	*hutH*	RBAM036400	histidine ammonia-lyase	56.41	56.76	5.18	5.2		C	0.47	-0.129	20
197	*iolA*	RBAM036770	methylmalonate-semialdehyde dehydrogenase	53.50	54.98	5.28	5.32		C	0.62	-0.138	21
198	*rocF*	RBAM037230	arginase	32.15	32.21	4.85	4.79		C	0.54	-0.185	17
199	*rocD*	RBAM037250	ornithine aminotransferase	43.70	40.93	5.27	5.38		C	0.61	-0.11	11
200	*leuD*	RBAM025310	isopropylmalate isomerase small subunit	23.11	22.47	4.91	4.98	+	C	0.63	-0.531	6
	Metabolism of carbohydrates and related molecules (80 different proteins)											
201	*dxs*	RBAM022600	1-deoxy-D-xylulose-5-phosphate synthase	69.67	68.32	5.99	6.51		C	0.56	-0.151	10
202	*lacG*	RBAM012180	putative 6-phospho-beta-galactosidase	53.84	54.27	5.46	5.66		C	0.61	-0.442	19

203	*pdhA*	RBAM014420	pyruvate dehydrogenase E1 alpha subunit	41.34	35.03	6.16	6.69	+	C	0.69	-0.458	8
204	*pdhB*	RBAM014430	pyruvate dehydrogenase E1 beta subunit	35.51	34.47	4.76	4.66	+	C	0.74	-0.014	12
205	*pdhC*	RBAM014440	pyruvate dehydrogenase (dihydrolipoamide acetyltransferase E2 subunit)	47.56	57.09	5.18	5.22	+	C	0.75	-0.34	18
206	*pdhD*	RBAM014450	dihydrolipoamide dehydrogenase E3 subunit	49.69	56.04	4.95	4.96		C	0.73	0.007	22
207	*pycA*	RBAM014720	pyruvate carboxylase	127.6	120.4	5.42	5.61	+	C	0.58	-0.304	22
208	*pyk*	RBAM026230	pyruvate kinase	61.83	70.08	5.14	5.14	+	C	0.68	-0.023	16
209	*pfkA*	RBAM026240	6-phosphofructokinase	34.24	34.28	6.23	6.73	+	C	0.65	-0.151	23
210	*pckA*	RBAM027580	phosphoenolpyruvate carboxykinase	58.04	57.11	5.36	5.31		C	0.57	-0.245	13
211	*pgi*	RBAM028430	glucose-6-phosphate isomerase	50.57	53.84	5.11	5.13	+	C	0.69	-0.352	11
212	*eno*	RBAM031260	enolase (2-phosphoglycerate dehydratase)	46.59	51.42	4.68	4.59	+	C	0.82	-0.243	18
213	*pgk*	RBAM031290	phosphoglycerate kinase	42.16	47.91	4.92	4.87	+	C	0.76	-0.089	15
214	*gapA*	RBAM031300	glyceraldehyde-3-phosphate dehydrogenase	35.76	35.02	5.36	5.51	+	C	0.86	-0.131	17
215	*tal*	RBAM034270	putative transaldolase	23.06	23.7	5.23	5.29	+	C	0.66	0.111	14
216	*fbaA*	RBAM034280	fructose-1,6-bisphosphate aldolase	30.37	31.93	5.26	5.39	+	C	0.78	-0.139	16
217	*tkt*	RBAM017690	transketolase	72.50	72.43	5.13	5.19	+	U	0.67	-0.261	21
218	*tpiA*	RBAM031280	triose phosphate isomerase	27.10	29.91	5	4.93	+	U	0.73	-0.17	14
219	*zwf*	RBAM022160	glucose-6-phosphate 1-dehydrogenase	55.47	54.68	5.36	5.4	+	C	0.62	-0.436	14
220	*yqjI*	RBAM022170	6-phosphogluconate dehydrogenase	51.71	49.91	5.19	5.19	+	C	0.68	-0.216	21
221	*gapB*	RBAM026060	glyceraldehyde-3-phosphate dehydrogenase	37.59	35.11	6.18	6.36		C	0.55	-0.223	13
222	*pgm2*	RBAM012200	phosphoglyceromutase	56.22	57.89	5.32	5.36	+	C	0.66	-0.315	19
223	*sacB*	RBAM037650	levansucrase	53.01	53.16	6.34	6.29		SpI	0.70	-0.667	23
224	*ptsH*	RBAM010230	phosphocarrier protein HPr component	9.18	17.4	4.86	4.69		SecP	0.80	-0.149	5
225	*iolB*	RBAM036770	inositol utilization protein B	30.42	34.09	5.03	5.01		SecP	0.54	-0.562	10
226	*yhxB*	RBAM009570	putative phosphomannomutase	64.15	72.04	5.1	5.15	+	SecP	0.58	-0.419	18
227	*gutB*	RBAM006540	sorbitol dehydrogenase (L-iditol 2-dehydrogenase)	38.28	47.87	5.56	5.73		C	0.53	-0.03	16
228	*ydjL*	RBAM006650	putative dehydrogenase	37.38	41.03	5.09	5.13	+	C	0.69	-0.044	12

229	*galE1*	RBAM012140	UDP-glucose 4-epimerase	36.39	32.96	5.96	6.41		C	0.56	-0.289	15
230	*oxdC*	RBAM020670	oxalate decarboxylase	43.52	45.2	5.18	5.02		C	0.58	-0.489	10
231	*xsa*	RBAM025570	alpha-N-arabinofuranosidase II	55.76	55.14	5.86	6.25		C	0.55	-0.455	16
232	*abfA*	RBAM025780	alpha-L-arabinofuranosidase I	56.87	57.06	5.64	6.16		C	0.53	-0.501	8
233	*araB*	RBAM025850	L-ribulokinase	61.43	58.21	5.56	6.04		C	0.53	-0.176	15
234	*ysdC*	RBAM025880	putative endo-1,4-beta-glucanase	39.35	38.61	5.63	5.86		C	0.58	-0.21	10
235	*ackA*	RBAM026400	acetate kinase	43.25	47.21	5.26	5.32	+	C	0.71	-0.048	22
236	*YtoP*	RBAM026970	conserved hypothetical protein	39.23	39.02	5.53	5.72		C	0.51	-0.356	15
237	*melA*	RBAM027220	alpha-D-galactoside galactohydrolase	48.85	51.31	5.94	6.4		C	0.54	-0.28	11
238	*yugK*	RBAM028460	putative NADH-dependent butanol dehydrogenase	43.43	39.37	4.81	4.73		C	0.66	-0.218	18
239	*yutF*	RBAM029400	conserved hypothetical protein	27.91	25.87	4.67	4.57		C	0.56	-0.137	8
240	*yvgN*	RBAM030570	putative dehydrogenase	31.53	32.12	5.11	5.11	+	C	0.65	-0.448	9
241	*gntK*	RBAM031430	gluconate kinase	56.69	56.55	5.92	6.31		C	0.52	-0.088	15
242	*yvkC*	RBAM032330	conserved hypothetical protein	94.34	91.02	4.82	4.76		C	0.53	-0.36	15
243	*alsD*	RBAM033160	alpha-acetolactate decarboxylase	28.46	29.82	4.66	4.39		C	0.68	-0.4	14
244	*alsS*	RBAM033170	acetolactate synthase	61.95	64	5.22	5.32		C	0.64	-0.114	13
245	*pta*	RBAM034850	phosphotransacetylase	34.74	34.17	4.77	4.59	+	C	0.68	-0.103	14
246	*licH*	RBAM035760	6-phospho-beta-glucosidase	48.62	54.09	5.28	5.32		C	0.54	-0.062	16
247	*ydhP*	RBAM035900	putative beta-glucosidase	54.32	52.46	5.9	6.36		C	0.55	-0.648	22
248	*galE*	RBAM036080	UDP-glucose 4-epimerase	36.88	34.57	5.34	5.39		C	0.56	-0.146	8
249	*bglH*	RBAM036340	beta-glucosidase	53.10	56.37	5.44	5.71		C	0.58	-0.544	17
250	*iolI*	RBAM036700	inositol utilization protein I	31.98	29.91	4.93	4.82		C	0.57	-0.275	10
251	*iolH*	RBAM036710	inositol utilization protein H	33.28	30.67	5.29	5.26		C	0.63	-0.453	17
252	*iolG*	RBAM036720	myo-inositol 2-dehydrogenase	38.11	41.04	5.14	5.13		C	0.56	-0.228	13
253	*iolE*	RBAM036740	inositol utilization protein E	33.78	29.81	4.84	4.69		C	0.52	-0.307	10
254	*iolC*	RBAM036760	inositol utilization protein C	36.00	38.82	5.37	5.49		C	0.57	-0.194	21
255	*iolS*	RBAM036800	inositol utilization protein S	34.79	34.59	5.17	5.14	+	C	0.62	-0.406	19

256	*acsA*	RBAM026800	acetyl-CoA synthetase	64.57	56.87	5.6	5.9	+	CM	0.56	-0.409	19
257	*ytiB*	RBAM027700	putative carbonic anhydrase	21.31	19.8	5.4	5.43		U	0.68	-0.206	7
258	*iolD*	RBAM036740	inositol utilization protein D	70.11	73.57	5.46	5.74		U	0.53	-0.292	19
259	*treA*	RBAM007980	trehalose-6-phosphate hydrolase	65.21	65.02	5.17	5.21		C	0.55	-0.708	18
260	*acoA*	RBAM008300	acetoin dehydrogenase E1 component (TPP-dependent alpha subunit)	35.94	35.08	5.03	4.91		C	0.60	-0.295	11
261	*glvA*	RBAM008360	maltose-6'-phosphate glucosid	50.60	53.83	4.93	4.87		C	0.57	-0.345	18
262	*yhfE*	RBAM010410	putative glucanase	38.59	47.35	5.93	6.33		C	0.53	-0.36	22
263	*galT1*	RBAM012130	galactose-1-phosphate uridyltransferase	56.52	56.73	6.24	6.71		C	0.57	-0.399	14
264	*galK1*	RBAM012150	galactokinase	42.82	44.42	5.62	6.01		C	0.55	-0.28	18
265	*pgm*	RBAM012200	predicted phosphatase/phosphohexomutase	25.59	25.15	4.95	4.81		C	0.54	-0.183	12
266	*dxr*	RBAM016390	1-deoxy-D-xylulose 5-phosphate reductoisomerase	42.42	49.02	5.29	5.39		C	0.56	-0.048	20
267	*yugJ*	RBAM028470	putative NADH-dependent butanol dehydrogenase	42.72	39.14	5.44	5.66	+	C	0.61	-0.194	13
268	*acoB*	RBAM008310	acetoin dehydrogenase E1 component (TPP-dependent beta subunit)	36.75	34.19	4.54	6.31		U	0.54	0.007	19
269	*pdxT*	RBAM000150	1-deoxy-D-xylulose-5-phosphate synthase	21.48	19.7	5.35	5.43		C	0.56	-0.151	15
270	*mdh*	RBAM026160	malate dehydrogenase	33.55	34.02	5.06	5.03	+	SpI	0.69	-0.009	19
271	*sdhA*	RBAM025510	succinate dehydrogenase (flavoprotein subunit)	65.03	65.31	5.8	5.92	+	SpI	0.60	-0.368	20
272	*sucC*	RBAM015920	succinyl-CoA synthetase (beta subunit)	41.49	41.02	5.01	5.01	+	C	0.67	-0.068	25
273	*sucD*	RBAM015930	succinyl-CoA synthetase (alpha subunit)	31.35	31.89	5.63	5.97	+	C	0.65	0.077	8
274	*citB*	RBAM017800	aconitate hydratase	99.20	99.47	5.01	5.09	+	C	0.67	-0.206	33
275	*odhB*	RBAM019120	dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex	45.39	56.36	5.12	5.11	+	C	0.61	-0.383	16
276	*sucA*	RBAM019130	2-oxoglutarate dehydrogenase E1 component	106.2	97.65	5.51	5.79	+	C	0.57	-0.472	24
277	*mleA*	RBAM021680	putative malolactic enzyme	45.86	55.27	5.09	5.17	+	C	0.56	0.087	11
278	*icd*	RBAM026170	isocitrate dehydrogenase	46.46	46.35	4.9	4.78	+	C	0.69	-0.242	18
279	*citZ*	RBAM026180	citrate synthase II	41.56	36.88	5.62	5.86		C	0.63	-0.28	14

280	*citG*	RBAM030160	fumarate hydratase II	50.57	52.33	5.55	5.75	+	C	0.63	-0.262	12
	Metabolism of coenzymes and prosthetic (18 different proteins)											
281	*nadE*	RBAM003360	NH3-dependent NAD+ synthetase	30.33	34.39	5.04	5.08		SecP	0.58	-0.567	17
282	*thiD*	RBAM035260	pyridoxal kinase	28.93	30.98	5.15	5.11		C	0.60	-0.012	9
283	*ykpB*	RBAM014180	thiamin biosynthesis homolog	33.32	30.8	8.35	6.87		C	0.57	-0.166	12
284	*pdxS*	RBAM000140	pyridoxine biosynthesis protein	31.59	31.85	5.25	5.29	+	C	0.73	-0.065	17
285	*hemE*	RBAM010350	uroporphyrinogen III decarboxylase	39.60	46.3	6.21	6.81		C	0.54	-0.212	12
286	*hemH*	RBAM010360	ferrochelatase	35.34	34.51	4.92	4.88		C	0.62	-0.55	10
287	*dhaS*	RBAM019060	aldehyde dehydrogenase	53.98	57.02	5.26	5.29		C	0.56	-0.196	11
288	*panC*	RBAM020570	pantoate-beta-alanine ligase	32.04	34.59	4.89	4.71		C	0.58	-0.253	11
289	*gsaB*	RBAM008800	glutamate-1-semialdehyde 2,1-aminomutase II	46.16	43.52	5.31	5.35		C	0.59	-0.044	18
290	*folD*	RBAM022640	bifunctional protein: methylenetetrahydrofolate dehydrogen-	30.60	27.79	6.19	5.89	+	C	0.62	-0.129	7
291	*hemB*	RBAM025190	delta-aminolevulinic acid dehydratase (porphobilinogen synthase)	36.23	34.73	5.36	5.41		C	0.60	-0.214	19
292	*yueK*	RBAM028830	putative nicotinate phosphoribosyltransferase	55.79	56.64	5.39	4.37		C	0.58	-0.406	22
293	*yueD*	RBAM028900	conserved hypothetical protein	26.93	26.3	5.28	5.39		C	0.52	-0.351	10
294	*yhxA*	RBAM009520	putative aminotransferase	49.33	40.87	5.54	5.74		C	0.52	-0.275	12
295	*dhbE*	RBAM029030	siderophore 2,3-dihydroxybenzoate/bacillibactin synthesis	59.68	56.87	5.45	5.72		C	0.55	-0.23	15
296	*menB*	RBAM027780	dihydroxynapthoic acid synthetase	29.95	27.98	5.48	5.41	+	C	0.67	-0.251	16
297	*dhbC*	RBAM029040	isochorismate synthase	43.02	53.32	5.27	5.35		C	0.51	-0.276	11
298	*dhbB*	RBAM029020	isochorismatase	34.28	36.08	4.74	4.61	+	U	0.56	-0.202	9
	Metabolism of lipids (24 different proteins)											
299	*yhaR*	RBAM010120	putative enoyl CoA hydratase	28.29	26.95	5.77	6.14		SecP	0.50	-0.15	15
300	*fabF*	RBAM011340	beta-ketoacyl-acyl carrier protein synthase II	43.69	49.57	5.25	5.41	+	SpI	0.64	-0.163	14
301	*ykwC*	RBAM013720	hypothetical oxidoreductase	30.69	27.13	5.51	5.73		SpI	0.56	-0.066	9
302	*fabL*	RBAM008740	enoyl-acyl carrier protein reductase	27.27	26.4	6.85	6.85		C	0.59	-0.066	7
303	*fabHB*	RBAM010400	beta-ketoacyl-acyl carrier protein synthase III	35.01	32.2	4.96	4.8	+	C	0.58	-0.03	11
304	*scoB*	RBAM018210	succinyl CoA:3-oxoacid CoA-transferase (subunit B)	24.18	26.5	5.46	5.65		C	0.49	-0.24	10

305	*yoxD*	RBAM018710	hypothetical oxidoreductase	25.80	26.27	5.26	5.41		C	0.66	-0.044	14
306	*accC*	RBAM022670	acetyl-CoA carboxylase subunit (biotin carboxylase subunit)	49.40	51.58	4.97	4.91	+	C	0.59	-0.163	21
307	*accA*	RBAM026250	acetyl-coenzyme A carboxylase carboxyl transferase subunit alpha	36.09	34.04	5.96	6.31	+	C	0.58	-0.296	16
308	*plsX*	RBAM015720	fatty acid/phospholipid synthesis protein	35.54	34.04	5.43	5.51		U	0.56	-0.035	9
309	*fabD*	RBAM015730	malonyl CoA-acyl carrier protein transacylase	34.29	34.09	4.88	4.71		U	0.57	-0.163	17
310	*yvaB*	RBAM030720	putative acyl carrier protein phosphodiesterase II	23.34	23.01	5.5	5.72		U	0.77	-0.39	12
311	*fabHA*	RBAM011330	3-oxoacyl-(acyl carrier protein) synthase III	33.70	31.9	5.06	5.04	+	C	0.56	-0.043	9
312	*fabI*	RBAM011730	enoyl-[acyl-carrier-protein] reductase	27.83	27.07	5.84	6.26	+	C	0.68	0.021	19
313	*bkdB*	RBAM022310	branched-chain alpha-keto acid dehydrogenase E2 subunit (lipoamide acyltransferase)	45.87	55.92	5.34	5.43		C	0.58	-0.366	17
314	*bkdAB*	RBAM022320	branched-chain alpha-keto acid dehydrogenase E1 subunit (2-oxoisovalerate dehydrogenase beta)	35.77	32.21	4.98	4.81		C	0.64	-0.035	15
315	*lpdV*	RBAM022340	branched-chain alpha-keto acid dehydrogenase E3 subunit (dihydrolipoamide dehydrogenase)	50.12	55.96	5.14	5.2		C	0.56	0.01	12
316	*buk*	RBAM022350	probable butyrate kinase	39.93	39.72	6.28	6.76		C	0.55	-0.179	17
317	*bcd*	RBAM022360	leucine dehydrogenase	39.66	47.43	5.11	5.12	+	C	0.61	-0.283	17
318	*accD*	RBAM026260	acetyl-CoA carboxylase (beta subunit)	32.20	34.13	5.75	6.32		C	0.59	-0.266	7
319	*yusJ*	RBAM029900	putative Acyl-CoA dehydrogenase	65.67	68.34	5.29	5.34		C	0.58	-0.261	24
320	*yusK*	RBAM029910	putative thiolase	40.91	41.03	5.83	6.26		C	0.51	-0.002	14
321	*bkdAA*	RBAM022330	branched-chain alpha-keto acid dehydrogenase E1 subunit (2-oxoisovalerate dehydrogenase alpha)	36.37	35.09	5.06	5.11		CM	0.59	-0.428	19
322	*accB*	RBAM022680	acetyl-CoA carboxylase subunit (biotin carboxyl carrier sub-unit)	17.17	18.87	4.62	5.78	+	U	0.66	-0.186	7
	Metabolism of nucleotides and nucleic acids (39 different proteins)											
323	*purA*	RBAM037450	adenylosuccinate synthetase	47.67	53.1	5.49	5.67	+	C	0.65	-0.318	10
324	*purK*	RBAM006850	phosphoribosylaminoimidazole carboxylase I	43.61	52.35	5.82	5.68		C	0.51	-0.223	17

325	*mtn*	RBAM013330	methylthioadenosine nucleosidase	25.15	27.03	5.16	5	+	C	0.66	0.047	13
326	*tmk*	RBAM000370	thymidylate kinase	23.81	23.01	5.69	5.8		C	0.54	-0.312	10
327	*yerA*	RBAM006960	putative adenine deaminase	66.55	65.76	5.87	6.37		C	0.53	-0.296	25
328	*pyrK*	RBAM015360	dihydroorotate dehydrogenase (electron transfer subunit)	27.88	32.03	6.54	6.81		C	0.49	-0.094	11
329	*hprT*	RBAM000790	hypoxanthine-guanine phosphoribosyltransferase	20.10	20.05	4.68	4.55		C	0.65	-0.035	15
330	*adk*	RBAM001620	adenylate kinase	24.09	25.95	4.78	4.72	+	C	0.63	-0.452	17
331	*guaA*	RBAM006760	GMP synthetase	57.63	56.99	4.86	4.83	+	C	0.68	-0.227	16
332	*purB*	RBAM006860	adenylosuccinate lyase	49.43	48.04	5.7	5.99	+	C	0.62	-0.367	24
333	*purC*	RBAM006870	phosphoribosylaminoimidazole succinocarboxamide synthetase	27.12	26.09	5.4	5.41	+	C	0.60	-0.374	22
334	*purM*	RBAM006920	phosphoribosylaminoimidazole synthetase	36.91	34.97	4.69	4.62	+	C	0.57	0.009	11
335	*purH*	RBAM006940	inosine-monophosphate cyclohydrolase	55.42	56.65	5.37	5.42	+	C	0.59	-0.202	19
336	*purD*	RBAM006950	phosphoribosylglycinamide synthetase	45.02	37.38	4.87	4.77	+	C	0.55	-0.041	10
337	*pyrC*	RBAM015330	dihydroorotase	46.63	50.19	5.39	5.44		C	0.57	-0.206	13
338	*pyrF*	RBAM015380	orotidine 5'-phosphate decarboxylase	26.06	26.12	5.61	5.79		C	0.52	-0.114	13
339	*nrdE*	RBAM017180	ribonucleoside-diphosphate reductase alpha subunit	80.57	74.89	5.72	6.18	+	C	0.65	-0.439	27
340	*deoD*	RBAM019420	purine nucleoside phosphorylase II (PNP II)	25.44	24.3	5.04	5.01	+	C	0.64	-0.114	19
341	*ndk*	RBAM020890	nucleoside diphosphate kinase	16.84	15.7	5.6	5.84	+	C	0.62	-0.24	12
342	*ypfD*	RBAM021020	putative 30S ribosomal protein S1	42.37	53.14	4.85	4.67	+	C	0.63	-0.289	19
343	*cmk*	RBAM021040	cytidylate kinase	24.90	28.6	5.28	5.3		C	0.56	-0.458	18
344	*punA*	RBAM021600	purine nucleoside phosphorylase	29.12	28.71	5.15	5.21		C	0.57	0.052	9
345	*drm*	RBAM021610	phosphopentomutase	43.94	53.59	5.19	5.19		C	0.60	-0.386	15
346	*guaC*	RBAM029180	GMP reductase	35.83	33.14	5.55	5.76		C	0.59	-0.164	11
347	*pyrG*	RBAM034300	CTP synthase	59.85	69.22	5.2	5.15	+	C	0.65	-0.288	20
348	*deoC*	RBAM036480	deoxyribose-phosphate aldolase	22.88	25.7	4.9	4.82		C	0.66	0.046	10
349	*purS*	RBAM006880	phosphoribosylformylglycinamidine synthetase	9.72	7.8	4.79	4.7		U	0.58	-0.323	6
350	*purQ*	RBAM006890	phosphoribosylformylglycinamidine synthetase	24.63	27.09	4.97	4.92	+	U	0.62	-0.039	11
351	*yhaM*	RBAM010170	putative CMP-binding factor	35.51	34.83	5.97	6.34	+	U	0.57	-0.282	18

352	*pyrH*	RBAM016350	uridylate kinase	26.08	27.02	5.28	5.26	+	U	0.63	-0.106	14
353	*guaB*	RBAM000120	inosine-monophosphate dehydrogenase	52.84	55.98	6.11	6.68	+	C	0.69	-0.182	11
354	*purL*	RBAM006850	phosphoribosylformylglycinamidine synthetase I	80.06	83.57	4.73	4.68	+	C	0.62	-0.138	12
355	*pyrB*	RBAM015320	aspartate carbamoyltransferase	33.79	33.12	6.22	6.41	+	C	0.54	-0.32	9
356	*pyrAA*	RBAM015340	carbamoyl-phosphate synthase pyrimidine-specificsmall chain	39.93	40.84	5.66	5.92	+	C	0.58	-0.245	16
357	*pyrAB*	RBAM015350	carbamoyl-phosphate synthase pyrimidine-specificlarge chain	118.0	110.4	5.05	5.09	+	C	0.54	-0.091	24
358	*gmk*	RBAM015510	putative guanylate kinase	23.35	24.78	4.73	4.68		C	0.61	-0.444	13
359	*pnpA*	RBAM016530	polynucleotide phosphorylase (PNPase)	77.60	76.08	5.03	5.07	+	C	0.66	-0.244	14
360	*nrdF*	RBAM017190	ribonucleoside-diphosphate reductase beta subunit	38.31	34.78	4.85	4.87		C	0.64	-0.416	8
361	*purF*	RBAM006910	glutamine phosphoribosylpyrophosphate amidotransferase	51.59	55.14	5.82	5.29	+	U	0.59	-0.121	13
	Metabolism of phosphate (1 protein)			0.00								
362	*ppaC*	RBAM037630	manganese-dependent inorganic pyrophosphatase	33.99	33.78	4.72	4.59	+	C	0.66	-0.126	15
	Metabolism of sulfur (3 different proteins)											
363	*sat*	RBAM015420	sulfate adenylyltransferase	42.60	50.21	5.38	5.56	+	C	0.60	-0.279	23
364	*yvgQ*	RBAM030600	putative sulfite reductase	64.74	62.22	6.25	6.82	+	C	0.58	-0.556	19
365	*yvgR*	RBAM030610	putative sulfite reductase (NADPH2) flavoprotein	67.39	71.22	4.82	4.76	+	C	0.54	-0.513	19
	Adaptation to atypical conditions (13 different proteins)											
366	*rsbR*	RBAM005010	positive regulator of sigma-B activity	30.70	28.03	4.86	4.72		C	0.54	0.088	6
367	*rsbT*	RBAM005030	serine/threonine-protein kinase	14.44	95.8	6.32	4.75		C	0.60	-0.256	6
368	*cspB*	RBAM009370	major cold-shock protein	7.25	7.3	4.63	4.4		C	0.94	-0.329	3
369	*clpC*	RBAM001110	class III stress response-related ATPase	89.96	81.44	5.79	6.19	+	C	0.61	-0.431	28
370	*ykoB*	RBAM013060	putative sigma-H activator	32.25	28.35	5.19	5.14		C	0.51	-0.218	8
371	*cspD*	RBAM020070	cold-shock protein	7.32	12.45	4.54	4.25		C	0.85	-0.471	4
372	*grpE*	RBAM023780	heat-shock protein	21.95	27.4	4.48	4.3	+	C	0.69	-0.871	12
373	*clpX*	RBAM025280	ATP-dependent Clp protease ATP-binding subunit (class III heat-shock protein)	46.22	54.89	4.77	4.75	+	C	0.67	-0.186	19

374	*clpP*	RBAM031850	ATP-dependent Clp protease proteolytic subunit (class III heat-shock protein)	21.82	20.8	4.96	4.9	+	C	0.70	-0.18	12
375	*htpG*	RBAM036820	class III heat-shock protein (molecular chaperone)	72.22	75.85	4.91	4.78	+	C	0.56	-0.589	26
376	*mcsB*	RBAM001100	modulation of CtsR repressionprotein	40.94	47.55	5.36	5.56		C	0.52	-0.294	9
377	*pspA*	RBAM006580	phage shock protein A homolog like protein	25.29	24.2	5.61	5.65		U	0.56	-0.69	8
378	*yacC*	RBAM000820	conserved hypothetical protein	31.84	33.21	4.77	4.69		C	**0.60**	-0.224	10
	Antibiotic production (17 different proteins)											
379	*pnbA*	RBAM031690	Para-nitrobenzyl esterase (intracellular esterase B)	52.82	56.86	4.86	4.82		Tat	0.55	-0.258	7
380	*RBAM_00981*	RBAM009810	putative beta-ketoacyl synthase	44.00	42.85	6.07	6.32		C	0.48	-0.224	15
381	*nrsD*	RBAM027440	acyl-carrier-protein	104.1	95.33	5.08	5.11		C	0.60	-0.265	24
382	*baeC*	RBAM016910	malonyl-CoA-[acyl-carrier-protein] transacylase	32.30	27.44	5.89	6.32		C	0.59	-0.275	11
383	*baeD*	RBAM016920	malonyl-CoA-[acyl-carrier-protein] transacylase	36.40	31.62	5.77	6.2	+	C	0.56	-0.215	17
384	*baeG*	RBAM016950	3-hydroxy-3-methylglutaryl CoA synthase	46.51	53.31	5.34	5.41		C	0.53	-0.228	21
385	*baeH*	RBAM016960	enoyl-CoA hydratase	29.18	30.02	5.48	5.67		C	0.53	-0.154	10
386	*baeI*	RBAM016970	enoyl-CoA-hydratase	27.77	27.43	5.93	6.33		C	0.54	-0.136	12
387	*bacD*	RBAM034890	bacilysin synthetase, amino acid ligase subunit	52.19	66.14	4.99	5.07	+	C	0.55	-0.173	22
388	*bacB*	RBAM034920	bacilysin synthetase B	26.85	27.01	5.22	4.69	+	U	0.65	-0.479	12
389	*dfnK*	RBAM021960	cytochrome P45	44.36	41.47	6.21	6.76	+	C	0.55	-0.435	18
390	*dfnA*	RBAM022080	acyltransferase/oxidoreductase	82.90	82.44	6.08	6.7		C	0.55	-0.297	18
391	*dfnY*	RBAM022070	hypothetical protein	36.72	38.41	5.95	5.21		C	0.50	-0.291	17
392	*mlnA*	RBAM014330	malonyl-CoA transacylase/oxidoreductase	86.27	51.33	5.8	6.4		C	0.54	-0.31	24
393	*fenE*	RBAM018420	fengycin synthetase	141.9	138.8	5.45	5.65		C	0.54	-0.295	19
394	*srfAD*	RBAM003690	surfactin synthetase D	27.74	26.01	5.88	6.32	+	C	0.52	-0.317	9
395	*srfAC*	RBAM003680	surfactin synthetase C	144.0	139.8	5.43	5.65		C	0.53	-0.362	16
	Detoxification (14 different proteins)											
396	*sodA*	RBAM023340	superoxide dismutase [Mn]	22.37	22.7	5.21	5.1	+	SecP	0.76	-0.491	7
397	*ksgA*	RBAM000510	dimethyladenosine transferase	32.83	31.61	5.73	6.19	+	C	0.51	-0.12	11

398	*yceD*	RBAM003160	putative tellurium resistance protein	20.69	22	4.47	5.2	+	C	0.67	-0.233	1
399	*yceE*	RBAM003170	putative tellurium resistance protein	20.87	22.01	4.57	5.2	+	C	0.57	-0.231	1
400	*yceH*	RBAM003200	putative toxic anion resistance protein	41.72	40.78	5.42	5.6		C	0.59	-0.649	13
401	*ynbB*	RBAM017240	conserved hypothetical protein	46.20	49.98	5.15	5.25		C	0.57	-0.155	10
402	*tpx*	RBAM026420	thiol peroxidase	18.15	18	4.99	4.75	+	C	0.69	-0.133	10
403	*cah*	RBAM003420	cephalosporin C deacetylase	35.56	34.87	5.61	5.75	+	U	0.55	-0.288	16
404	*ycsF*	RBAM004280	putative lactam utilization protein	27.08	27.81	5.38	5.45		C	0.56	-0.031	11
405	*yceC*	RBAM003150	putative tellurium resistance protein	21.59	21.9	5.1	5.2		C	0.62	-0.217	11
406	*ahpC*	RBAM036960	alkyl hydroperoxide reductase subunit C (small subunit)	20.51	20.5	4.51	4.29	+	C	0.87	-0.228	9
407	*ahpF*	RBAM036960	alkyl hydroperoxide reductase (large subunit) and NADH dehydrogenase	54.83	55.17	4.8	4.76	+	C	0.67	-0.098	21
408	*mlnI*	RBAM014410	putative penicillin binding protein	41.09	35.65	5.62	5.89		CM	0.59	-0.13	19
409	*yvaK*	RBAM030910	putative carboxylesterase	28.33	28.62	5.42	5.67	+	C	0.58	-0.518	15
	Phage-related functions (1 protein)											
410	*yobO*	RBAM017590	conserved hypothetical protein	86.68	89.21	5.23	5.27		SecP	0.55	-0.302	14
	No similarity (5 different proteins)											
411	*RBAM012210*	RBAM012210	hypothetical protein	44.74	52.69	5.42	5.62		SecP	0.61	-0.474	14
412	*RBAM_00746*	RBAM007460	hypothetical protein	36.87	31.88	5.63	5.96		C	0.61	-0.19	14
413	*RBAM011990*	RBAM011990	hypothetical protein	38.21	34.15	5.32	5.51		C	0.63	-0.205	12
414	*RBAM037120*	RBAM037120	hypothetical protein	69.69	73.87	5.19	5.19		C	0.57	-0.582	17
415	*RBAM007470*	RBAM007470	hypothetical protein	50.35	48.13	5.6	5.87		C	0.59	-0.333	17
	Similar to unknown proteins from *B. subtilis* (43 different proteins)											
416	*ykzG*	RBAM014280	hypothetical protein	8.26	8.01	4.78	4.68		SecP	0.61	-0.72	6
417	*yulF*	RBAM028270	conserved hypothetical protein	36.58	36.13	5.6	5.72		SecP	0.58	-0.255	15
418	*yhgC*	RBAM010330	hypothetical protein	18.70	19.99	5.6	5.67		SecP	0.54	-0.525	8
419	*yukJ*	RBAM028990	conserved hypothetical protein	25.43	26.88	4.92	4.8		SecP	0.57	-0.577	11
420	*ykgB*	RBAM012860	conserved hypothetical protein	38.54	38.48	5.42	5.59		SecP	0.61	-0.444	15

421	*ysaA*	RBAM025980	conserved hypothetical protein	30.07	28.01	5.06	4.71	+	C	0.56	-0.367	12
422	*yurU*	RBAM029790	conserved hypothetical protein	52.74	57.15	5.1	5.13	+	U	0.72	-0.447	17
423	*ycsE*	RBAM004270	conserved hypothetical protein	28.16	27.57	4.71	4.58		C	0.50	-0.304	7
424	*yabN*	RBAM000670	conserved hypothetical protein	56.32	56.79	4.7	4.63		C	0.55	-0.411	15
425	*yitU*	RBAM011130	conserved hypothetical protein	30.52	28.74	5.24	5.31		C	0.53	-0.333	15
426	*ycjH*	RBAM011920	conserved hypothetical protein	27.61	27.05	5.73	5.83		C	0.51	-0.48	5
427	*ytnP*	RBAM027000	hypothetical protein	31.89	34.02	5.64	6.06		C	0.59	-0.541	13
428	*yvqH*	RBAM030250	conserved hypothetical protein	25.95	31.89	6.3	6.81		C	0.60	-0.94	10
429	*yacK*	RBAM001130	conserved hypothetical protein	40.65	46.93	5.7	6.12		C	0.53	-0.121	8
430	*ybfQ*	RBAM002740	conserved hypothetical protein	37.53	38.51	5.14	5.07		C	0.66	-0.692	19
431	*yheA*	RBAM010030	conserved hypothetical protein	13.56	12.3	4.71	4.6		C	0.66	-0.677	13
432	*yhfK*	RBAM010460	hypothetical protein	22.75	26.9	5.68	5.91		C	0.52	-0.148	8
433	*yitL*	RBAM011040	conserved hypothetical protein	32.12	35.57	4.96	4.91		C	0.55	-0.497	8
434	*yitS*	RBAM011080	conserved hypothetical protein	31.13	28.62	5.62	5.92		C	0.50	-0.078	13
435	*yitV*	RBAM011140	conserved hypothetical protein	29.29	26.8	5.6	5.6	+	C	0.57	-0.258	15
436	*yjcF*	RBAM011900	conserved hypothetical protein	19.46	19.1	5.38	5.6		C	0.59	-0.453	10
437	*ykaA*	RBAM012700	conserved hypothetical protein	23.78	24.1	5.08	5.09	+	C	0.61	-0.378	13
438	*ylaL*	RBAM014680	hypothetical protein	17.56	19.19	5.14	5.14		C	0.60	-0.404	4
439	*ypgR*	RBAM020020	conserved hypothetical protein	42.77	49.9	5.03	5.07		C	0.57	-0.571	15
440	*engA*	RBAM020990	putative GTP binding protein	48.71	52.35	5.55	5.79		C	0.54	-0.166	13
441	*ytqI*	RBAM026310	conserved hypothetical protein	35.11	35.78	4.93	4.9	+	C	0.54	-0.313	3
442	*ytpQ*	RBAM026940	conserved hypothetical protein	30.87	31.02	5.16	5.11		C	0.53	-0.493	12
443	*yvfW*	RBAM031460	conserved hypothetical protein	52.90	54.07	5.83	5.79		C	0.48	-0.417	11
444	*ywfI*	RBAM034860	conserved hypothetical protein	29.47	27.85	5.26	5.34	+	C	0.63	-0.423	14
445	*yxjG*	RBAM036120	conserved hypothetical protein	42.83	47.87	5.51	5.71	+	C	0.60	-0.501	21
446	*ydjI*	RBAM006610	conserved hypothetical protein	37.16	41.03	5.18	5.13		U	0.61	-0.403	12
447	*yllA*	RBAM014980	conserved hypothetical protein	62.55	64.48	5.47	5.78	+	U	0.55	-0.47	25

448	*yurX*	RBAM029780	conserved hypothetical protein	48.26	55.91	5.3	5.25	+	U	0.65	-0.315	11
449	*ydjG*	RBAM006590	hypothetical protein	38.31	34.64	4.99	5.04		C	0.50	-0.252	13
450	*YdcI*	RBAM005090	conserved hypothetical protein	80.87	82.43	6.11	6.72		C	0.52	-0.432	17
451	*yerI*	RBAM007040	conserved hypothetical protein	38.89	34.71	5.18	5.29		C	0.57	-0.557	9
452	*yeeI*	RBAM007230	conserved hypothetical protein	26.35	32.01	4.51	4.31		C	0.60	-0.433	11
453	*yfkA*	RBAM008180	conserved hypothetical protein	43.13	48.13	5.82	6.11		C	0.56	-0.497	12
454	*ykqC*	RBAM014270	conserved hypothetical protein	61.40	57.05	6.01	6.47	+	C	0.60	-0.124	29
455	*engC*	RBAM015610	putative GTPase	33.09	34.97	5.83	6.17		C	0.55	-0.325	11
456	*yxeH*	RBAM036500	conserved hypothetical protein	30.10	28.01	4.84	4.72		C	0.55	-0.154	8
457	*ygxA*	RBAM008840	hypothetical protein	34.49	28.78	5.62	5.89		U	0.55	-0.24	13
458	*ylbP*	RBAM014960	conserved hypothetical protein	18.88	18.9	4.9	4.83		U	0.55	-0.671	5
	Similar to unknown proteins from other organisms (3 different proteins)											
459	*RBAM017620*	RBAM017620	conserved hypothetical protein	30.63	27.87	6.07	6.41		SpI	0.58	-0.442	15
460	*RBAM005550*	RBAM005550	hypothetical protein	18.87	18.8	4.93	4.8		C	0.56	-0.366	7
461	*RBAM028450*	RBAM028450	hypothetical protein RBAM02913	23.15	21.92	4.62	4.48		C	0.61	0.014	7

a) MW theor. is abbreviation of theoretical molecular weight.
b) MW observ. is abbreviation of observed molecular weight.
c) p*I* theor. is abbreviation of theoretical isoelectric point.
d) p*I* obser. is abbreviation of observed isoelectric point.
e) asterix marks proteins present in the proteome of cells grown in minimal medium.
f) Prediction of the theoretical cellular protein localization. Abbreviations used: C, cytoplasmic protein; CM, cytoplasmic membrane; U, localization unknown; putative secreted protein predicted by SignalP, LipoP and TatP are denoted SpI, SpII or Tat.; SecP, non-classical secreted protein predicted by SecretomeP.
g) CAI, is abbreviation of Codon Adaptation Index.
h) GRAVY, is abbreviation of Grand Average of Hydropathy.

Table 4 *B. amyloliquefaciens* proteins exclusively expressed by the cells grown in minimal medium. Proteins were identified by MALDI-MS and MS/MS on the 2D gel of analytical window p*I* 4-7.

	Gene	ID	Protein function / similarity	MW theor.[a]	p*I* theor.[b]	Cellular localization[c]	CAI[d]	GRAVY[e]	Peptide count
	Metabolism of amino acids and related molecules								
1	*aroE*	RBAM020760	3-phosphoshikimate 1-carboxyvinyltransferase	45.02	5.4	C	0.548	-0.027	18
2	*leuA*	RBAM025340	2-isopropylmalate synthase	56.92	5.51	C	0.536	-0.283	28
3	*leuB*	RBAM025330	3-isopropylmalate dehydrogenase	39.20	5.05	C	0.547	-0.095	10
4	*leuC*	RBAM025320	isopropylmalate isomerase large subunit	52.10	6.14	C	0.546	-0.334	17
5	*alaT*	RBAM028500	hypothetical protein RBAM_028500	41.23	5.09	C	0.515	-0.019	6
6	*argD*	RBAM011220	acetylornithine aminotransferase	40.99	5.84	C	0.531	-0.057	24
7	*argF*	RBAM011250	ornithine carbamoyltransferase	35.26	5.33	C	0.493	-0.198	19
8	*argG*	RBAM026380	hypothetical protein RBAM_026380	44.60	5.06	C	0.592	-0.139	12
9	*carA*	RBAM011230	carbamoyl phosphate synthase small subunit	38.67	5.69	U	0.550	-0.191	11
10	*carB*	RBAM011240	carbamoyl phosphate synthase large subunit	113.22	5.03	C	0.552	-0.133	29
11	*goxB*	RBAM011680	glycine oxidase	40.85	6.03	C	0.526	0.284	19
12	*hisF*	RBAM032080	imidazole glycerol phosphate synthase subunit HisF	27.20	5.46	C	0.543	-0.118	8
13	*hisI*	RBAM032070	bifunctional phosphoribosyl-AMP cyclohydrolase/phosphoribosyl-ATP pyrophosphatase protein	23.22	5.19	C	0.526	-0.46	14
14	*hisZ*	RBAM032140	ATP phosphoribosyltransferase regulatory subunit	43.20	5.42	C	0.507	-0.19	12
15	*lysC*	RBAM025540	aspartate kinase	43.45	4.78	C	0.561	0.002	18
16	*trpA*	RBAM020790	tryptophan synthase subunit alpha	29.10	5.19	U	0.599	0.09	17
17	*trpB*	RBAM020800	tryptophan synthase subunit beta	43.55	5.65	C	0.583	-0.155	16
18	*trpC*	RBAM020820	indole-3-glycerol-phosphate synthase	27.94	5.14	C	0.522	-0.14	19
19	*trpE*	RBAM020840	anthranilate synthase component I	57.77	5.41	C	0.556	-0.259	18
20	*ybgE*	RBAM002760	branched-chain amino acid aminotransferase	40.05	5.33	C	0.474	-0.199	17
21	*yjcI*	RBAM011930	cystathionine gamma-synthase	41.74	5.07	C	0.581	-0.221	10
22	*yjcJ*	RBAM011940	cystathionine beta-lyase	42.27	5.92	C	0.514	-0.118	16

23	*yclM*	RBAM004040	putative homoserine dehydrogenase	50.27	5.73	C	0.519	-0.184	31
	Metabolism of carbohydrates and related molecules; Specific pathways								
24	*yoaC*	RBAM018750	putative xylulokinase	53.46	5.94	U	0.571	-0.129	10
	Metabolism of coenzymes and prosthetic groups								
25	*nadA*	RBAM024900	quinolinate synthetase	41.33	5.71	C	0.520	-0.322	9
26	*nadC*	RBAM024910	nicotinate-nucleotide pyrophosphorylase	30.93	5.61	C	0.504	-0.196	17
27	*thiC*	RBAM009060	thiamine biosynthesis protein	65.90	5.3	C	0.538	-0.438	18
28	*thiG*	RBAM011700	thiazole synthase	26.95	4.86	C	0.512	0.129	17
29	*yjbV*	RBAM011720	phosphomethylpyrimidine kinase	28.87	5.93	C	0.487	-0.123	13
	Biotin biosynthesis								
30	*bioA*	RBAM018280	adenosylmethionine--8-amino-7-oxononanoate transaminase	49.99	5.64	C	0.572	-0.183	18
31	*bioD*	RBAM018260	dithiobiotin synthetase	25.52	6.06	C	0.513	-0.02	14
	Riboflavin biosynthesis								
32	*ribH*	RBAM021390	riboflavin synthase subunit beta	16.21	5.43	C	0.585	0.155	12
33	*fabZ*	RBAM033570	(3R)-hydroxymyristoyl-ACP dehydratase	15.64	5.52	C	0.584	-0.145	7
	Metabolism of nucleotides and nucleic acids								
34	*pyrD*	RBAM015370	dihydroorotate dehydrogenase 1B	33.00	5.77	C	0.517	0.14	9
35	*upp*	RBAM034050	uracil phosphoribosyltransferase	23.05	6.23	C	0.637	-0.108	18
	Adaptation to atypical conditions								
36	*rsbV*	RBAM005050	anti-anti-sigma factor (antagonist of RsbW)	11.94	4.78	C	0.524	0.003	3
	Miscellaneous								
37	*ybaL*	RBAM001800	ATP-binding mrp-like protein	38.32	5.45	C	0.571	-0.101	12
	Transport/binding proteins and lipoproteins								
38	*ssuB*	RBAM009110	aliphatic sulfonate ABC transporter (binding protein)	30.22	8.61	CM	0.445	-0.291	8
39	*yqiZ*	RBAM022260	putative amino acid ABC transporter (ATP-bindingprotein)	26.66	5.67	CM	0.547	-0.229	10
	Similar to unknown proteins from *B. subtilis*								
40	*ybgG*	RBAM002780	homocysteine methyltransferase	34.36	5	C	0.524	-0.159	7

41	*yitJ*	RBAM011020	bifunctional homocysteine S-methyltransferase/5,10-methylenetetrahydrofolate reductase protein	68.32	5.69	C	0.526	-0.247	32
42	*yqiW*	RBAM022290	conserved hypothetical protein	15.93	5.01	C	0.585	-0.378	5
43	*yvbK*	RBAM031150	conserved hypothetical protein	17.49	4.96	C	0.542	-0.271	7
	Protein synthesis								
44	*mtnA*	RBAM013330	methylthioribose-1-phosphate isomerase	38.83	4.85	C	0.505	-0.057	13
45	*mtnK*	RBAM013340	methylthioribose kinase	44.73	5.34	C	0.563	-0.319	16
	RNA modification								
46	*miaA*	RBAM017130	tRNA delta(2)-isopentenylpyrophosphate transferase	35.37	6.36	U	0.504	-0.35	3
	RNA synthesis; Regulation								
47	*ccpA*	RBAM026860	hypothetical protein RBAM_026860	36.93	5.08	C	0.586	-0.181	10
48	*tenA*	RBAM011660	transcriptional activator	27.17	4.83	C	0.619	-0.479	14

a) MW theor. is abbreviation of theoretical molecular weight.
b) p*I* theor. is abbreviation of theoretical isoelectric point.
c) Prediction of the theoretical cellular protein localization. Abbreviations used: C, cytoplasmic protein; U, localization unknown; CM cytosolic membrane.
d) CAI, is abbreviation of Codon Adaptation Index.
e) GRAVY is abbreviation of Grand Average of Hydropathy.